Evolution of Cooperation and Virulence in an Opportunistic Pathogen

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Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich

von

Elisa Teresa Granato

aus
Deutschland

Promotionskommission

Prof. Dr. Rolf Kümmerli (Vorsitz)

Prof. Dr. Adrian Hehl

Prof. Dr. Jakob Pernthaler

Zürich 2017

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Dissertation

for
Obtainment of a Scientific Doctorate
(Dr. sc. nat.)

submitted to the
Faculty of Mathematics and Natural Sciences
of the
University of Zurich

by

Elisa Teresa Granato

from
Germany

Promotion Committee

Prof. Dr. Rolf Kümmerli (Chairman)

Prof. Dr. Adrian Hehl

Prof. Dr. Jakob Pernthaler

Zurich 2017

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1 ACKNOWLEDGEMENTS

To adequately express my gratitude to everyone involved is beyond the scope of this document, but I will try my best.

I would like to thank …

Rolf Kümmerli, for your support, motivation, enthusiasm, and seemingly infinite patience throughout the years. Your optimism and steady guidance were essential to the success of my PhD, and any future students (I am sure there will be many!) can consider themselves lucky to have you as a supervisor. Your advice and ideas have shaped my development as a scientist and will surely resonate for years to come.

Freya Harrison, for the great collaboration and for introducing me to Twitter. Adin Ross-Gillespie for statistical support and being a great office mate.

My past and current PhD committee members Jakob Pernthaler, Adrian Hehl and Otto Cordero, for useful feedback and fair criticism on the projects. Judith Zingg for organizing a flawless graduate school experience.

Martin Ackermann for taking the time to directly supervise my Master's project and writing a million reference letters, and for valuable advice on how to navigate academia without burning out.

Julia Vorholt for hosting my first steps in a Swiss lab and being supportive in times of conflict. Hans-Martin Fischer for teaching me all about cloning, and for your infectious enthusiasm about lab work.

Thorsten Mascher and Susanne Gebhard for introducing me to the magical world of microbes all the way back in "Mikrobiologie 1".

Julian for inspiring me to keep running, for introducing me to the best game in the world, and for being my favorite human.

My father Nunzio for always asking questions about microbes, my mother Eva for psychological guidance, and both of my parents for love and support.

My brother Luca for tech support, and for all the giggles. My sister-in-law Steffi for beating me to the first "Dr. Granato" and showing me how it's done. My nieces Teresa and Sara for being the future.

Christoph for the daily PhD chats, pupper pictures and highest quality sequencing. Enrico for excellent sushi and hours of MarioParty. Tine for always teaching me how to language, and for being the best friend in the world. Sofia for career support and "leaning in" with me. Ruben for the early years, and for stats advice in the cafeteria. Otze, Baste, Kleine and Groβe for the good old times and upholding the *LMU Qualitätssiegel*.

Anne, Michael, Elena, Marta, Chiara, Santosh, Subham, Alex, Jos and Selina for support, friendship and teamwork in the lab, and the members of the Eberl group for the great working atmosphere.

And finally, Kami and Furnando for unconditional affection.

2 SUMMARY

Cooperation is ubiquitous in nature. Two (or more) parties working together can generate great benefits for both sides, and numerous examples for complex forms of collaboration exist in humans and other animals. However, cooperative systems can be vulnerable to exploitation by "cheats", which do not contribute, but nevertheless reap the benefits of cooperation. How cooperative behaviors might have evolved in light of this risk of cheating is therefore an important research topic in evolutionary biology.

Historically, social behaviors were mainly investigated in "higher animals" such as vertebrates and insects. However, by now we have realized that even single-celled bacteria socially engage with each other, and can thus be used to study cooperation. A good example for collaboration among bacteria is the production of iron-scavenging siderophores: these molecules are secreted by bacterial cells when iron is scarce, bind to extracellular iron and can then be taken up by any cell in the surrounding environment with a matching receptor. In the bacterial pathogen *Pseudomonas aeruginosa*, it was shown that siderophore non-producing "cheats", which do not produce siderophores but can use those made by others, can invade a population of producers and destabilize the cooperative system. Since siderophores and other secreted metabolites are important for virulence in many bacterial pathogens, it was suggested that these social dynamics would also impact the evolution of virulence. In my thesis, I investigated different aspects of cooperative behaviors in bacteria, using siderophore production in *P. aeruginosa* as a model system. I particularly focused on how infections are influenced by social interactions among pathogens.

During an infection, cells of *P. aeruginosa* cooperatively secrete the siderophore pyoverdine into the host environment to scavenge iron.

Pyoverdine has been regarded as an important virulence factor for decades, but until now it was unclear whether its importance might vary depending on the infection context. To address this, I performed a meta-analysis on published "survival" experiments in different hosts with *P. aeruginosa* infections (Project 1). I found that although pyoverdine is important for infections, it is often not essential. These findings highlight that virulence is influenced by many factors, a fact that might complicate efforts to identify the most important virulence factors. A meta-analysis as I have conducted here, can help to navigate through this complex subject.

My results from Project 1 also show that bacterial infections are highly context-dependent. In order to investigate the evolution of pathogens, a more holistic approach is therefore needed, taking into account the potential influence of different environments. Towards this, I conducted an experimental evolution (Project 2) to assess how *P. aeruginosa* adapts to conditions inside and outside of a host organism, and how adaptation would affect virulence towards the same host. I let wild type bacteria evolve with and without a host, the nematode *Caenorhabditis elegans*, while also varying the degree of spatial structure in the environments. I found that virulence dropped dramatically when mutant bacteria lost virulence traits that were either i) not needed outside of the host, or ii) reduced due to social exploitation. These results demonstrate that the social context of virulence factors must be considered to understand the evolution of bacterial pathogens.

We know that social exploitation by evolved cheats is common for cooperative traits in bacteria. However, so far it was not clear what happens after a cheat has spread through a population of cooperators. Would cheats evolve back to being cooperators when left to grow on their own? To answer this question, I investigated two such "cheat" strains with reduced pyoverdine production in Project 3. Both strains were allowed to evolve in different environments predicted to promote (or

hinder) cooperation. Subsequently, I measured pyoverdine production in evolved clones to test whether natural selection had steered them towards more or less cooperation. I could not observe evolution of increased pyoverdine production, but instead production was further reduced under conditions where it was most beneficial, suggesting continued exploitation of the cooperative trait. Thus, the conditions necessary for the evolution of cooperation seem to be more stringent than previously thought.

3 ZUSAMMENFASSUNG

Kooperation, oder Zusammenarbeit zwischen Individuen, ist in der Natur in vielen verschiedenen Lebewesen anzutreffen. Wenn zwei (oder mehrere) Parteien zusammenarbeiten, kann dies einen grossen Nutzen für alle Beteiligten bringen. In uns Menschen - und in vielen anderen Tieren - finden sich zahlreiche Beispiele für komplexe Formen von Kooperation. Harmonische Zusammenarbeit birgt aber oft auch eine Kehrseite: das Auftreten von „Schmarotzern", die nichts zur allgemeinen Kooperation beitragen, aber trotzdem den Nutzen davontragen. Wie sich kooperative Verhalten trotz dieser Gefahr der Ausbeutung im Laufe der Evolution durchsetzen konnten, ist deshalb ein wichtiges Forschungsthema in der Evolutionsbiologie.

Ursprünglich wurden solche sozialen Verhaltensweisen vor allem in „höheren Tieren" - wie zum Beispiel Wirbeltieren und Insekten - untersucht. Mittlerweile wissen wir aber, dass selbst einzellige Bakterien miteinander kooperieren und für die Erforschung der Kooperation gut geeignet sind. Ein gutes Beispiel für Zusammenarbeit zwischen Bakterienzellen ist die Produktion eisenbindender Siderophore. Wenn Eisen stark limitiert ist, werden diese Siderophore sekretiert, binden danach an extrazelluläres Eisen und können anschliessend von allen Zellen in der näheren Umgebung aufgenommen werden, welche einen passenden Rezeptor besitzen. Siderophore stellen in diesem Zusammenhang ein sogenanntes „öffentliches Gut" dar. In dem bakteriellen Krankheitserreger *Pseudomonas aeruginosa* wurde gezeigt, dass "Schmarotzer"-Zellen, welche selbst keine Siderophore produzieren, aber die Siderophore anderer Produzenten benutzen können, in einer Population von Produzenten überhandnehmen können und das Kooperationssystem aus der Balance bringen. Da Siderophore und andere sekretierte Stoffe in vielen bakteriellen Krankheitserregern wichtig für die Virulenz sind, könnte diese soziale Dynamik auch die Evolution der Virulenz beeinflussen. In

meiner Dissertation habe ich verschiedene Aspekte kooperativer Verhaltensweisen in Bakterien anhand der Siderophorproduktion in *P. aeruginosa* untersucht. Besonderes Augenmerk legte ich darauf, wie Infektionen durch soziale Interaktionen zwischen Pathogenen beeinflusst werden.

Während einer Infektion sekretieren *P. aeruginosa*-Zellen gemeinschaftlich das Siderophor Pyoverdin in die Wirtsumgebung, um Eisen aufzunehmen. Es ist seit langem bekannt, dass Pyoverdin ein wichtiger Virulenzfaktor ist. Bisher war allerdings unklar, ob dessen Bedeutung je nach Infektionskontext variiert. In Projekt 1 führte ich deshalb eine Meta-Analyse durch, und analysierte publizierte „Survival"-Experimente in verschiedenen Wirtstieren mit *P. aeruginosa* Infektionen. Meine Untersuchung ergab, dass Pyoverdin für Infektionen zwar wichtig, aber oft nicht essentiell ist. Dieses Ergebnis zeigt, dass Virulenz von vielen Faktoren beeinflusst wird, was die Suche nach den wichtigsten Virulenzfaktoren erschweren kann. Eine Meta-Analyse, wie ich sie hier durchgeführt habe, kann die Bearbeitung dieser komplexen Thematik erheblich erleichtern.

Meine Ergebnisse aus Projekt 1 zeigen auch, dass bakterielle Infektionen sehr kontextabhängig sind. Um die Evolution von Krankheitserregern zu untersuchen, ist deshalb ein ganzheitlicher Ansatz von Nöten, der den Einfluss verschiedener Infektions- und Umweltbedingungen mit einbezieht. In Projekt 2 habe ich dazu eine experimentelle Evolution durchgeführt, und untersucht, wie sich *P. aeruginosa* an verschiedene Bedingungen innerhalb und ausserhalb eines Wirtstiers anpasst, und wie diese Anpassungen die Virulenz der Bakterien im selben Wirt beeinflussen. Während der Evolution entwickelten sich die Bakterien entweder mit oder ohne einem Wirtstier - dem Fadenwurm *Caenorhabditis elegans* – und die Umweltbedingungen varriierten hinsichtlich ihrer räumlichen Struktur. Die Virulenz ist immer dann drastisch gesunken, wenn mutierte Bakterien Virulenzfaktoren verloren haben, die entweder i) außerhalb des Wirts nicht

benötigt oder ii) durch soziale Ausbeutung reduziert wurden. Hier konnte ich also zeigen, dass der soziale Kontext von Virulenzfaktoren sehr wichtig ist, um die Evolution bakterieller Krankheitserreger zu verstehen.

Es ist bekannt, dass sich soziale „Schmarotzer"-Bakterien in verschiedenen kooperativen Systemen entwickeln können. Unklar war aber bislang, was passiert nachdem sich ein solcher Schmarotzer in einer Population von kooperativen Zellen ausgebreitet hat. Können Schmarotzer durch Evolution wieder zur Zusammenarbeit gebracht werden, wenn sie auf sich alleine gestellt sind? Um diese Frage zu beantworten, hab ich in Projekt 3 zwei solche „Schmarotzer"-Stämme mit reduzierter Pyoverdinproduktion untersucht. Beide Stämme durften sich unter verschiedenen Umweltbedingungen entwickeln, welche kooperative Verhaltenweisen begünstigen (beziehungsweise hemmen) sollten. Anschließend habe ich die Pyoverdinproduktion in evolvierten Klonen gemessen, um zu testen ob die natürliche Selektion zu mehr oder weniger Kooperation geführt hat. Ich konnte keine Evolution hin zu mehr Pyoverdinproduktion beobachten. Stattdessen wurde die Produktion gerade dann weiter reduziert, wenn sie den grössten Nutzen brachte. Dies deutet auf eine kontinuierliche Ausbeutung des kooperativen Systems hin. Die Bedingungen für die Evolution von Kooperation scheinen demnach begrenzter zu sein als bislang angenommen.

4 GENERAL INTRODUCTION

"In the beginning the universe was created. This has made a lot of people very angry and was widely regarded as a bad move."

~Douglas Adams, The Hitchhiker's Guide to the Galaxy (1979)

4.1 Cooperation in microorganisms

Sociality is ubiquitous throughout all domains of life [1,2]. Bacteria are no exception to this: in natural environments, microbes commonly exist in communities comprised of multiple strains and species with whom they engage in a variety of complex social interactions [3]. The constant struggle for nutrients and space experienced by community-dwelling bacteria has led to the evolution of diverse mechanisms to kill competing cells, e.g. the production of toxins and antibiotics [4], or to benefit from other cells' presence by cooperating with them [5]. In general, cooperation can be broadly defined as any action that benefits individuals other than the actor, and have been - at least partially - selected for that purpose [6]. According to Hamilton [7], cooperative traits can be further subdivided into i) mutualism, when the behavior benefits both the actor and the recipient, and ii) altruism, when the behavior is costly for the actor and beneficial for the recipient (Fig. 1).

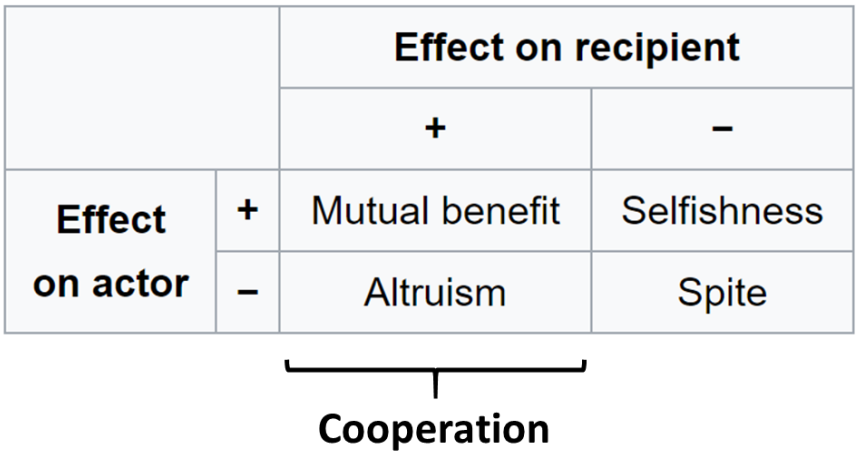

Figure 1. Hamilton's classification of the four types of social behaviors. Hamilton [7] categorized social behaviors according to whether the resulting consequences for actor and recipient are beneficial (increase direct fitness) or costly (decrease direct fitness). Figure adapted from West *et al*. [6].

4.1.1 Mutualism – "You scratch my back and I'll scratch yours"

In mutualistic interactions, partners benefit from each other's activities. Individuals partaking in the interaction often have a shared interest in cooperation, and both partners get direct fitness benefits from acting cooperatively [8]. A well-known example for mutualism between different species is the pollination of plants by various insects, where both partners benefit from the interaction [9]. In microbes, the cooperative production of so-called "public goods" falls into this category. Bacteria produce a number of different factors that are released into the extracellular environment and perform a function outside of the cell. The benefits from this function, e.g. nutrient release, can often be accessed by any neighboring cell. In a fully mutualistic scenario, all cells in a population share both the costs and the benefits of secretion by producing - more or less – the same amount of public good [10]. If there is considerable variation, or even bimodality in the production among cells of the same population, then a public good trait can become altruistic (see section 4.1.2).

One example of mutualistic behavior in bacteria is cell-to-cell-communication through the secretion of signaling molecules, so called "quorum sensing". Many bacteria secrete auto-inducer molecules into the environment, triggering a response in the cells once a certain threshold concentration – the "quorum" – is reached [11]. These sensing mechanisms are most often used to coordinate the expression of traits that are beneficial at high cell densities [12–14](but see also [15] for an example of a low-density switch). In bacterial pathogens, many secreted virulence factors are under the control of quorum sensing systems [16,17].

Another mutualistic behavior common in bacteria is the secretion of enzymes and secondary metabolites used for nutrient acquisition: proteases extracellularly digest proteins into short-chained peptides to facilitate nutrient uptake [18], invertases are enzymes that digest sucrose

outside of yeast cells [19], and siderophores scavenge iron from the environment [20,21]. The latter will be introduced in detail in section 4.3.

Other examples for microbial mutualism include the cooperative production of polymers that form the extracellular matrix in biofilms [22], social motility through the secretion of biosurfactants [23], and predation in groups [24].

4.1.2 Altruism – the ultimate sacrifice

In an altruistic interaction, an organism performs a behavior that provides a direct fitness cost to itself but is beneficial to the recipient. Workers in social insect colonies engage in altruism by foregoing their own reproduction and instead helping their close relatives to reproduce, often a single queen [25]. Whether a given trait is mutualistic or altruistic often depends on the specific context [6], and only a few examples for altruism exist in microorganisms.

One such case can be found in the slime mold *Dictyostelium discoideum*, a unicellular eukaryote [26]. Upon starvation, cells come together to form multicellular aggregates called fruiting bodies, consisting of a nonviable stalk that makes up roughly 20 % of all cells, and a spherical tip of spores [10,27]. Since the stalk cells are nonviable, they effectively forego reproduction in favor of the spore cells in an act of altruism [28,29]. A similar form of altruistic fruiting body formation can be found in the soil bacterium *Myxoccocus xanthus* [30]. There, some cells within a fruiting body develop into stress-resistant spores, whereas the others undergo autolysis or remain undifferentiated [31,32]. Another example along these lines is programmed cell death in the brewer's yeast *Saccharomyces cerevisiae*, which has also been suggested to represent an altruistic behavior [33,34].

In other system, extreme forms of cooperation are independent from nutrient starvation, but are prompted by infection. In several bacterial species, cells respond to phage attacks by triggering suicide in infected cells [35,36]. This benefits the surrounding cells by preventing further transmission of the parasite, and was suggested to represent a form of bacterial altruism [37,38].

4.1.3 Evolution and maintenance of microbial cooperation

Traditional Darwinian evolutionary theory is based on the idea that organisms will engage in behaviors that maximize their own fitness, and avoid behavior that accrues fitness benefits to others. From this point of view, explaining the evolutionary origins of cooperative interactions, whether mutualistic or altruistic, has been a great challenge and a highly debated topic in evolutionary biology [6,8,39–52]. When Charles Darwin developed his theory of natural selection, he recognized that cooperative behaviors such as altruism pose a "special difficulty" to his framework, even referring to them as potentially "fatal to the whole theory" [53]. The development of inclusive fitness theory and "Hamilton's rule" has added a crucial piece to the puzzle by laying out that individuals can gain both direct fitness benefits - through their own reproduction - and indirect fitness benefits - through the reproduction of individuals with shared genes [7]. According to Hamilton's rule, genes encoding a cooperative trait increase in frequency if $r*B > C$, where C is the cost to the actor performing cooperation, B is the benefit gained by the individual receiving cooperation and r is the relatedness between the actor and the recipient. Importantly, "relatedness" at the genetic level in this context refers to relatedness at the locus encoding the cooperative trait, and does not necessarily correspond to shared ancestry [5]. This is especially true for microbial communities, where horizontal gene transfer is widespread [54]. The evolution of cooperative behaviors can be

facilitated by maximizing the likelihood of interacting with a related individual, and several mechanisms have been suggested to increase relatedness.

Kin recognition - the differential treatment of related individuals compared with unrelated individuals – represents one way of ensuring that a cooperative act is preferentially geared towards related individuals. In microbes, kin recognition often involves specific biochemical interactions between a receptor and an identification molecule [55], and was suggested to facilitate the evolution of several cooperative traits [56].

Another way to ensure that cooperative benefits mostly accrue to related individuals, is through limited dispersal. Limited dispersal (high spatial structure) leads to asexually reproducing microbes being mostly surrounded by clonemates. This increase in relatedness can result in high indirect fitness benefits for cooperating cells [57–59], but it can also increase local competition among relatives [60]. Likewise, we know that high relatedness can stabilize extreme forms of cooperation (see section 4.1.2), such as altruistic fruiting body formation [61,62]. On the other hand, it was also demonstrated that altruism can evolve even when relatedness is low, as long as the costs for the altruistic act are low as well [37]. Factors favoring cooperation are therefore highly context-dependent, and their importance differs between different cooperative traits and environments.

Once a cooperative trait has evolved and spread in a population, it can potentially be destabilized by the evolution of non-cooperative "cheats". These individuals still receive the benefits from the remaining cooperators, but without paying any of the costs of the cooperative behavior [63]. Cheats gain a fitness advantage over the cooperators, and can spread through a population of cooperators under certain circumstances [64–68]. In extreme cases, the whole cooperative system can collapse,

because a critical fraction of the population does not cooperate anymore, resulting in population decline and extinction [69,70].

Examples of cheats outcompeting cooperators can be found in quorum-sensing systems, where both the act of signaling itself and the response to the signal carry a substantial metabolic cost for the signaling cell, which can be exploited by cheats [16,71,72]. Other systems known to be prone to cheat invasion are the cooperative secretion of siderophores [73–75] and proteases [76], and fruiting body formation in *D. discoideum* [28].

Despite these well-studied examples of exploitation, cooperation is still widespread in nature, and a number of factors have been suggested to prevent social collapse in the presence of cheats [2]. By channeling fitness benefits preferentially to cooperators, high degrees of spatial structure have been shown to limit the ability of cheating mutants to invade [67,77,78]. When cooperation is based on the secretion of public good molecules, high spatial structure has the added side-effect of limiting the diffusion of secreted molecules [79]. This renders public goods effectively more "private", and thus less accessible to non-producing cells [76,80]. In contrast to this, well-mixed environments with high diffusion rates are known to facilitate cheat invasion [67].

Aside from spatial structuring, the composition of a given population plays a big role in predicting cheat success. For example, cheats are fitter when population density is high [19,65,81] due to being physically closer to secreted public goods. Similarly, cheats can gain great advantages when they are rare, but not when they occur at higher frequencies in the population, because fewer public goods are then available for each individual cheat [66,82].

Another factor known to prevent cheat invasion is genetic linkage of the cooperative trait with another, non-social trait. For example, if there is a regulatory connection between cooperation and another metabolic process, an evolved non-cooperator can potentially suffer from a net

disadvantage. These pleiotropic effects were shown to prevent cheat invasion in bacteria [83,84] and social amoebae [85].

As outlined in section 4.1.1, the secretion of shareable metabolites is important for many aspects of microbial life, such as nutrient uptake, motility and predation. In bacterial pathogens, these secreted metabolites also mediate crucial parts of the infection process, such as damage to the host tissue. Any factors that influence cooperative traits could consequently also affect virulence traits. In my thesis, I investigated this link between cooperation and virulence using a model bacterial pathogen, which I will introduce in the next section.

4.2 Virulence in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that is metabolically highly versatile and can cause serious infections in different organisms, including humans [86–90]. It is widely regarded as one of the most troublesome opportunistic human pathogens [91–93]. Reasons for its notoriety include its ability to colonize and thrive in a vast number of artificial and natural environments [94], its high levels of intrinsic drug resistance [95], and its propensity to cause serious infections in the immunocompromised [96] and in patients with existing medical conditions such as cystic fibrosis [97,98] and burn wounds [99].

Like many opportunistic pathogens, *P. aeruginosa* typically only becomes pathogenic in response to a perturbation in the host, such as a wound or a disease, and is often acquired by the host from an environmental reservoir [100,101]. Unlike some other pathogens, it is not specialized on a specific host species or genus. Instead, its host range includes different species of plants [88,102], invertebrates [103–105] and mammals, including humans [86,92,106].

4.2.1 Virulence factors - a recipe for infections

Virulence factors (VFs) can be broadly defined as any trait that enables a pathogen to cause infection. These traits can be associated with colonization of the host – e.g. surface attachment to the host tissue –, immunoevasion, immunosuppression, damage to the host tissue, or nutrient acquisition during an infection. The generalist strategy of pathogenicity in *P. aeruginosa* is facilitated by a comparatively large genome [107] that harbors a whole arsenal of chromosomally encoded VFs [17,108,109]. As secondary metabolites, the role of VFs is highly context-dependent and their production is usually tightly controlled by multiple regulatory systems [110]. Many of them are under quorum-sensing control and are therefore only produced when high cell densities are reached [17].

Some of the most well-described and important VFs in *P. aeruginosa* include: the siderophores pyoverdine and pyochelin, used for iron-scavenging in the host [103,106]; different proteases to digest host tissue [111–113]; pyocyanin and other phenazine toxins to damage host cells through their redox-activity [114–116]; Type III secretion systems to infect effector proteins into host cells [117]; cyanide to inhibit respiration in host cells [118,119]; and biofilm formation to protect bacteria from immune responses and antimicrobial treatments [120,121].

4.2.2 (Social) Evolution of virulence

Bacterial pathogens are a major cause of morbidity and mortality in humans [122–125], and understanding how pathogens adapt to the environments they occupy is crucial in finding better ways to manage them. Theoretical approaches to studying pathogen evolution are often based on the assumption that a single, obligate pathogen infects a single host organism on which it is specialized [126,127]. However, none of these

assumptions hold true for the majority of bacterial pathogens: most are non-obligate opportunists that are able to infect a wide range of different hosts without being specialized on a single genus, species or organ [128].

Part of the complexity in studying opportunists lies in the fact that they adapt both to the host and to the non-host environment [129,130]. In the host, they may adapt to avoid immune responses and increase their ability to colonize host tissues [131–134]. They can also undergo selection in the non-host, abiotic environment, such that any resulting effects in their ability to cause infections could be accidental [129].

Finally, social interactions between pathogens, ranging from cooperation to competition, can influence the evolution of virulence [135–139]. Social interactions determine the extent to which pathogens compete for resources, which in turn affects their ability to colonize and harm the host. In the context of an infection, many secreted virulence factors can be regarded as cooperatively produced public goods, because they perform a function outside of the pathogen cell - e.g. digestion of host tissue - which can then benefit all cells in the local surroundings, e.g. by releasing nutrients that are then freely available [21,140,141].

As outlined in section 4.1.3, these cooperative traits can sometimes be exploited by non-producers. In *P. aeruginosa*, it was shown that this exploitation can have extensive consequences both for within-host pathogen growth and damage to the host [103,140,142,143]. A virulence trait that has received much attention in this context is the production of siderophores in *P. aeruginosa*, which I will now explain in more detail.

4.3 Siderophores and the struggle for iron

Iron (Fe) is an essential element for almost all forms of life, as it is crucial for many enzymatic processes, respiratory metabolism and DNA and RNA synthesis [144]. Siderophores are powerful iron chelators secreted by bacteria and other microorganisms in order to acquire iron from their surroundings [145]. For free-living bacteria in the environment, iron is usually present in its insoluble ferric form Fe^{3+}, making it metabolically inaccessible [146]. In the case of bacterial pathogens infecting a host organisms, iron is often bound to iron-sequestering host proteins [147,148]. Therefore, specialized iron-uptake mechanisms such as siderophores are necessary to overcome iron starvation [149]. Bacterial siderophores display a high diversity among species [150–152], and even among strains of the same species [153]. Generally, siderophores are actively secreted into the environment where they bind to iron, and the iron-siderophore complex is then taken up again by the cell. In gram-negative bacteria, Fe-siderophore recognition and uptake relies on outer-membrane receptors usually encoded in the siderophore biosynthesis clusters [152]. While most siderophore receptors only recognize one specific type, some bacterial strains can take up several different siderophores by simultaneously producing different receptors [154,155].

4.3.1 Siderophores in *P. aeruginosa*

P. aeruginosa produces two main siderophores, pyoverdine and pyochelin [156,157]. Pyoverdine is regarded as the main siderophore in this context, as its affinity for iron is several orders of magnitude higher than that of pyochelin [156,157]. It was suggested that *P. aeruginosa* dynamically switches between these two iron-uptake system, depending on the severity of iron limitation [158].

Pyoverdine is synthesized via non-ribosomal peptide synthesis, with at least 14 different enzymes being involved in its production and secretion [159]. This process is metabolically very costly and consequently under tight regulation (Fig. 2). Under iron-limited conditions, the alternative sigma factor PvdS is expressed and upregulates the biosynthesis of pyoverdine [156], which is then secreted by an ATP-dependent efflux pump [159,160].

Once secreted, pyoverdine molecules bound to iron are actively taken up and transported into the periplasm by a TonB-dependent transporter [156]. In the periplasm, iron is then released from pyoverdine and moved into the cytoplasm through an ATP-binding cassette transporter [159,160]. The free pyoverdine molecule is then secreted again, meaning that this costly molecule can be re-used several times [161]. Once iron becomes more abundant, the Ferric Uptake Regulator (FUR) protein prevents pyoverdine synthesis by binding to the promoter region of *pvdS*, inhibiting its transcription [156].

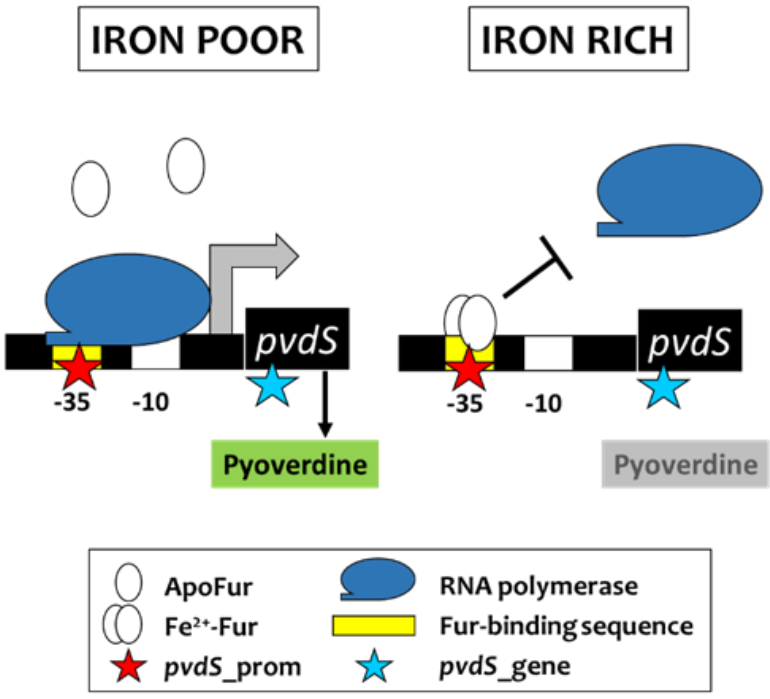

Figure 2. Schematic representation of pyoverdine regulation by the iron-starvation sigma factor PvdS and Fur. Under iron replete conditions, the Fe^{2+}-Fur complex binds to the *pvdS* promoter region and prevents its transcription. When iron is scarce, Fur cannot bind to the promoter, and RNA polymerases can freely transcribe *pvdS*. The PvdS protein then upregulates pyoverdine production.

4.3.2 Siderophores and their role in virulence

During a bacterial infection, the infected host organism will mount a physiological reaction to inhibit bacterial growth as part of the innate immune response [162]. Iron is then usually sequestered by host proteins like transferrin in order to restrict access to this essential element [147,148]. This leads to host tissue and infection sites being highly iron limited environments, which requires pathogens to secrete siderophores in order to retain the ability to grow [163]. Some bacteria are able to secrete siderophores that are not recognized by the immune system, so called "stealth siderophores", which facilitate immune evasion [164]. In *P. aeruginosa*, both siderophores (pyoverdine and pyochelin) are known to be involved in virulence in acute infections, with pyoverdine likely playing the dominant role due to the severity of iron limitation during an infection [106]. PvdS, the main regulator for pyoverdine production, is also involved in the regulation of other virulence factors, such as proteases and a toxin, further contributing to the importance of pyoverdine to virulence [165,166].

The importance of pyoverdine for acute bacterial infections was demonstrated in a variety of different hosts, from plants [88] and invertebrates like nematodes and insect larvae [103–105], to mammals [86,106]. In chronic infections, e.g. in the lungs of patients with cystic fibrosis, *P. aeruginosa* tends to lose the ability to produce pyoverdine over time [167–170]. It was suggested that this loss is due to social interactions in the lung, where pyoverdine non-producers appear through mutation from the background of the initial infecting strain, and outcompete the pyoverdine producers through social exploitation of the shared pyoverdine molecules [143]. But there are also non-social effects that could explain the loss of pyoverdine production in chronic infections. For example, bacteria could switch to alternative iron uptake systems, such as pyochelin, heme or citrate [134,167,171,172]. Furthermore, pyoverdine

could be lost because free iron becomes more abundant in lungs with increased levels of tissue damage [173]. When iron becomes more bioavailable, costly siderophore production could then be counter-selected.

4.3.3 Siderophores and social interactions

Once a siderophore molecule is secreted it diffuses freely in the environment, and iron-bound siderophores can then be taken up by any cell with a suitable receptor. Importantly, the receiving cell is not guaranteed to be the original producing cell. In a population of siderophore-producing bacteria, this leads to a common pool of siderophores that are produced cooperatively, and the costs of production as well as the benefits of uptake are shared among neighboring cells [21]. These interactions can be exploited by cells that do not engage in siderophore production, but still reap the benefits of uptake [73,74].

Since siderophores are very common in the microbial world and their regulation and molecular properties have been described in great detail for many species [149,156], they have become a model trait to study bacterial cooperation and exploitation [5]. In this context, the siderophores enterochelin [75], pyochelin [84] and - particularly – pyoverdine [174,175], have received the most attention. In *P. aeruginosa*, pyoverdine non-producing "cheat" bacteria have been shown to i) evolve readily from a producing ancestor [64]; and ii) outcompete cooperating strains in mixed culture in a density- and frequency-dependent manner [65,66]. Since pyoverdine usage relies on diffusion and pyoverdine production is metabolically costly (see above), environmental conditions are known to influence the likelihood of successful exploitation. The competitive advantage of pyoverdine non-producers over producers in mixed culture was shown to be maximized

when environments are well-mixed and molecules diffuse readily (low spatial structure) and when the costs and benefits of pyoverdine production are high, i.e. when iron is strongly limited [67,73]. Most studies on *P. aeruginosa* social interactions and cheating have been conducted using clinical isolates, but siderophore exploitation was recently also demonstrated in natural *Pseudomonas* isolates from non-clinical samples [176].

4.4 Aims of this thesis

The three chapters of this thesis address different aspects of cooperative behaviors in bacteria, using siderophore production in the opportunistic pathogen *P. aeruginosa* as a model system.

In Project 1, I conducted a meta-analysis on published survival data in different host organisms infected by *P. aeruginosa*. My aim was to quantify the consequences of cooperation in bacterial pathogens on the infected hosts. During an infection, *P. aeruginosa* cells cooperatively secrete the siderophore pyoverdine into the host environment in order to scavenge iron. Because iron is both necessary for bacterial growth and generally scarce in the host, pyoverdine has been regarded as an important virulence factor for decades. However, it is unclear whether it is truly essential for any given infection, or whether its importance might vary depending on the specific infection context.

In Project 2, I investigated the evolution of virulence in a host-pathogen system using experimental evolution. My goal was to test how virulence is affected by adaptation to different abiotic environments and a host, and to explore the role of social evolution in this context. Towards this, I propagated *P. aeruginosa* in unstructured or structured environments in the presence or absence of its host *Caenorhabditis elegans*, and

subsequently conducted phenotypic screens for changes in virulence and virulence factor production.

In Project 3, I examined whether cheats can revert back to being cooperators when environmental conditions change. I conducted experimental evolution in different environments predicted to promote (or hinder) cooperation, and tested whether natural selection steered bacteria towards lower or higher investment into a cooperative trait. I used mutant *P. aeruginosa* strains with low levels of pyoverdine production, and let them evolve in environments with different degrees of spatial structure and iron availability. Subsequently, I measured changes in pyoverdine production in evolved clones to test for reversion to full cooperation.

5 PROJECT 1 – Virulence Factors

"A couple of months in the laboratory can frequently save a couple of hours in the library."

Frank Westheimer, American chemist (1912-2007)

5.1 Do Bacterial "Virulence Factors" Always Increase Virulence? A Meta-Analysis of Pyoverdine Production in *Pseudomonas aeruginosa* As a Test Case

This research was published in Frontiers in Microbiology.

Granato, E. T., Harrison, F., Kümmerli, R., Ross-Gillespie, A. Do Bacterial 'Virulence Factors' Always Increase Virulence? A Meta-analysis of Pyoverdine Production in *Pseudomonas aeruginosa* as a Test Case.

Frontiers in Microbiology. 2016 (7): 1-13.

doi: 10.3389/fmicb.2016.01952

Supplementary Tables S1-S4 are too large to be included in this document, and instead are available for download here:

http://journal.frontiersin.org/article/10.3389/fmicb.2016.01952/full

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 December 2016
doi: 10.3389/fmicb.2016.01952

Do Bacterial "Virulence Factors" Always Increase Virulence? A Meta-Analysis of Pyoverdine Production in *Pseudomonas aeruginosa* As a Test Case

Elisa T. Granato [1], Freya Harrison [2], Rolf Kümmerli [1]* and Adin Ross-Gillespie [1,3]*

[1] Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland, [2] School of Life Sciences, University of Warwick, Coventry, UK, [3] SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland

OPEN ACCESS

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***Correspondence:**
Elisa T. Granato
egranato@botinst.uzh.ch
Rolf Kümmerli
rolf.kuemmerli@uzh.ch

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: *08 August 2016*
Accepted: *21 November 2016*
Published: *12 December 2016*

Citation:
Granato ET, Harrison F, Kümmerli R and Ross-Gillespie A (2016) Do Bacterial "Virulence Factors" Always Increase Virulence? A Meta-Analysis of Pyoverdine Production in Pseudomonas aeruginosa As a Test Case. Front. Microbiol. 7:1952. doi: 10.3389/fmicb.2016.01952

Bacterial traits that contribute to disease are termed "virulence factors" and there is much interest in therapeutic approaches that disrupt such traits. What remains less clear is whether a virulence factor identified as such in a particular context is also important in infections involving different host and pathogen types. Here, we address this question using a meta-analytic approach. We statistically analyzed the infection outcomes of 81 experiments associated with one well-studied virulence factor—pyoverdine, an iron-scavenging compound secreted by the opportunistic pathogen *Pseudomonas aeruginosa*. We found that this factor is consistently involved with virulence across different infection contexts. However, the magnitude of the effect of pyoverdine on virulence varied considerably. Moreover, its effect on virulence was relatively minor in many cases, suggesting that pyoverdine is not indispensable in infections. Our works supports theoretical models from ecology predicting that disease severity is multifactorial and context dependent, a fact that might complicate our efforts to identify the most important virulence factors. More generally, our study highlights how comparative approaches can be used to quantify the magnitude and general importance of virulence factors, key knowledge informing future anti-virulence treatment strategies.

Keywords: virulence factor, siderophore, pyoverdine, meta-analysis, virulence

INTRODUCTION

Understanding which bacterial characteristics contribute most to disease is a major area of research in microbiology and infection biology (Rahme et al., 1995; Jimenez et al., 2012; Lebeaux et al., 2014). Bacterial characteristics that reduce host health and/or survival are considered "virulence factors." Such factors include structural features like flagella and pili that facilitate attachment to host cells (Josenhans and Suerbaum, 2002; Kazmierczak et al., 2015), as well as secreted products like toxins and enzymes that degrade host tissue (Vasil et al., 1986; Lebrun et al., 2009), or siderophores that scavenge iron from the host (Miethke and Marahiel, 2007). Research on virulence factors has not only increased our fundamental understanding of the mechanisms underlying virulence, but has also identified potential novel targets for antibacterial therapy. There is indeed much current interest in developing "anti-virulence" drugs to disrupt virulence factor production—the idea being

that by simply disarming pathogens rather than killing them outright, we could ostensibly elicit weaker selection for drug resistance (Clatworthy et al., 2007; Pepper, 2008; Rasko and Sperandio, 2010).

Although our understanding of different types of virulence factors and their interactions is continuously deepening, it is still unclear just how generalizable this assembled knowledge is. It is often assumed, for reasons of parsimony, that a given structure or secreted molecule central to the virulence of a particular bacterial strain in a specific host context will similarly enhance virulence in another bacterial strain, or in a different host (Dubern et al., 2015). Yet, ecological theory predicts that the effects of a given trait will frequently vary in response to the environment (Lambrechts et al., 2006). In the context of infections, this may be particularly true for opportunistic pathogens, which face very heterogeneous environments: they can live in environmental reservoirs (e.g., soil, household surfaces), as commensals of healthy hosts, or, when circumstances allow, as pathogens, causing serious infections in a range of different hosts and host tissues (Kurz et al., 2003; He et al., 2004; Calderone and Fonzi, 2001). Opportunistic pathogens underlie many hospital-acquired infections, especially in immune-compromised patients (Gaynes and Edwards, 2005; Obritsch et al., 2005; Länger and Kreipe, 2011), and the treatment of such infections is often challenging because, as generalists, such pathogens are pre-selected to be tenacious and highly adaptable. Thus, in designing new anti-virulence drugs against opportunistic pathogens, we need to know not only whether the targeted trait is indeed associated with pathogenicity, but also the generality of this association across different pathogen strain backgrounds, host species, and infection types.

To address this issue, we introduce a meta-analysis approach, which allows us to quantify variation and overall effects of virulence factors across host environments. As a test case, we focus on pyoverdine, a siderophore secreted by the opportunistic pathogen *Pseudomonas aeruginosa* to scavenge iron from the host environment (Visca et al., 2007). **Table 1** provides an overview of the workflow of our meta-analysis, where we combined the outcomes of 81 individual virulence experiments from 24 studies (Meyer et al., 1996; Takase et al., 2000; Xiong et al., 2000; Gallagher and Manoil, 2001; Ochsner et al., 2002; Silo-Suh et al., 2002; Salunkhe et al., 2005; Harrison et al., 2006; Attila et al., 2008; Papaioannou et al., 2009; Zaborin et al., 2009; Carter et al., 2010; Nadal Jimenez et al., 2010; Oliver, 2011; Romanowski et al., 2011; Feinbaum et al., 2012; Okuda et al., 2012; Imperi et al., 2013; Kirienko et al., 2013; Ross-Gillespie et al., 2014; Dubern et al., 2015; Lin et al., 2015; Lopez-Medina et al., 2015; Minandri et al., 2016, see also Tables S1, S2 in the Supplemental Material). Using a weighted meta-analysis approach, we were able to investigate the evidence for pyoverdine's contribution to virulence across eight host species, including vertebrates, invertebrates and plants, five tissue infection models and various *P. aeruginosa* genotypes. We chose pyoverdine production as the model trait for our analysis because: (i) it has been extensively studied across a range of *Pseudomonas* strains (Meyer et al., 1997); (ii) its virulence effects have been examined in a large number of host species; (iii) *P. aeruginosa* is one of the most troublesome opportunistic human pathogens, responsible for many multi-drug resistant nosocomial infections (Hauser and Rello, 2003; Hirsch and Tam, 2011); and (iv) multiple anti-virulence drugs have been proposed to target pyoverdine production and uptake (Kaneko et al., 2007; Imperi et al., 2013; Ross-Gillespie et al., 2014). The applied question here, then, is whether targeting pyoverdine could generally and effectively curb pathogenicity.

MATERIALS AND METHODS

Literature Search

We conducted an extensive literature search, using a combination of two online databases: Web of Science and Google Scholar. The following terms were used to search abstracts and full texts: "aeruginosa" in combination with "pyoverdin" or "pyoverdine" and in combination with "virulence" or "infection" or "pathogen" or "disease" or "mortality" or "lethality." This search was first performed on May 19th 2014 and it was repeated periodically until Aug 1st 2016 in order to include more recent publications. In addition, the reference lists of all shortlisted studies were scanned for relevant publications. We further contacted the corresponding authors of several publications to ask for unpublished datasets. Ultimately, no unpublished datasets were included in the final meta-analysis (see Supplementary Tables S1+S3 for details).

Inclusion Criteria

The database search yielded a total of 529 studies, and we identified 10 additional records through other sources. These 539 studies were then scanned for relevant content according to the following set of inclusion criteria. Studies were considered potentially eligible for inclusion if they contained original research, were written in English and provided data that compared the virulence of a wildtype pyoverdine-producing *P. aeruginosa* strain with that of a mutant strain demonstrating impaired pyoverdine production. We defined virulence as a decrease in host fitness, measured as an increase in mortality or tissue damage when infected with bacteria. We defined "wildtype strains" as strains that were originally clinical isolates, have been widely used in laboratories as virulent reference strains and have not been genetically modified. Strains with impaired pyoverdine production included strains that were completely deficient in pyoverdine production and strains that were only partially deficient, i.e., that produced less than the wildtype strain under identical experimental conditions. We considered both genetically engineered knock-out strains and clinical isolates with reduced pyoverdine production. Since pyoverdine is not directly encoded in the genome but is synthesized via non-ribosomal peptide synthesis (Visca et al., 2007), there are no studies that modified the functioning of pyoverdine through targeted mutagenesis. There were 32 original publications containing 120 experiments that satisfied our criteria and were thus considered appropriate for in-depth examination.

We screened these 120 experiments using a second set of rules to identify those experiments that contain comparable quantitative data, which is essential for a meta-analysis. Inclusion criteria were: (i) virulence was measured directly (and not

TABLE 1 | Meta-analysis workflow for this study.

In general	This study	Details
1. Formulate hypothesis and predictions	HYPOTHESIS: Pyoverdine is an important virulence factor for *Pseudomonas aeruginosa*. PREDICTION: Pyoverdine-defective mutants cause less virulence than wildtype strains.	Tables S1+S2
2. Systematically search for relevant studies	We searched (see details in main text) for any reports of experiments featuring monoclonal infections of whole live host organisms with *P. aeruginosa strains* known to vary in pyoverdine phenotype, where virulence was quantified in terms of host mortality.	
3. Extract and standardize effect sizes and their standard errors	For each case reporting host survival, we calculated the (log) ratio of mortality odds from pyoverdine-mutant infections vs. wildtype infections—i.e., the (log) odds-ratio.	
4a. Check heterogeneity across studies	Our assembled effect sizes were more heterogeneous than expected from chance—even when we allowed that some of this variation could be due to random noise.	Figure 1
4b. Consider putative moderator variables (optional)	We tested for evidence of distinct sub-groups in our dataset, within which the effects might be more homogeneous. We identified four putative moderators and codified each study for the following: (i) host taxon; (ii) infection type; (iii) strain background; and (iv) level of pleiotropy expected, given the particular mutation(s) involved.	
4c. Check for publication bias (optional)	We found that smaller/lower-powered studies were more likely to report large effect sizes in support of the hypothesis, whereas larger/higher-powered studies tended to report smaller effect sizes.	Figure 2
5. Derive mean effect size(s); quantify influence of moderator variables (if applicable)	Despite the steps taken (see above), our dataset still showed substantial heterogeneity. Estimates of mean effect sizes (in/across subgroups) and moderator coefficients should therefore be viewed as best approximations.	Figure 3

inferred indirectly via genetic analysis); (ii) virulence was measured *in vivo* (and not *in vitro* via virulence factor production); (iii) virulence was measured quantitatively as direct damage to the host caused by bacterial infections, and not by indirect or qualitative measures such as bacterial growth performance in the host, threshold infective dose required to kill a host, the damage associated with virulence factor administration, or resistance to macrophage-like predation (Ryan et al., 2009; Jeukens et al., 2014; Kirienko et al., 2015); and (iv) absolute virulence data were presented (and not only data scaled relative to the wildtype without information on the absolute risk of mortality, since effect sizes cannot be calculated from such data). This second set of rules was fulfilled by 24 original publications containing 81 individual experiments (see Tables S1, S2 in the Supplemental Material). For an overview of the whole selection process, see Figure S1 in the Supplemental Material.

Data Extraction and Effect Size Calculations

From all of these 81 experiments, we extracted information on: (i) the host organism; (ii) the type of infection; (iii) the observation period of infected hosts; (iv) the identity of the control (wildtype) strain; (v) the identity of the pyoverdine-deficient strain; (vi) the mutated gene in the pyoverdine-defective strain; (vii) the mutation type (e.g., insertion/deletion); (viii) the sample size used for the wildtype and mutant experiments; and (ix) the relevant virulence measure (host survival or tissue damage) for wildtype and mutant strains (see Table S1 in the Supplemental Material). This information was used to categorize the experiments and identify potentially important moderator variables (see below).

Next, we extracted quantitative data from these experiments so we could calculate effect sizes for the virulence associated with pyoverdine production. For mortality assays, we extracted raw counts of how many individuals (i.e., whole animals or seedlings) died and how many survived following infection with a dose of *P. aeruginosa* wildtype or, alternatively, a mutant strain known to be deficient for pyoverdine production. For experiments on tissue damage, we extracted information on the number of individuals with and without the symptoms related to tissue damage (e.g., a lesion in an organ). In cases with zero counts (i.e., either all or none of the individuals in a particular treatment group died or experienced tissue damage), we converted counts to 0.5 to avoid having zero denominators in the subsequent calculations of the (log-odds ratio) effect sizes (Cox, 1970). In cases where data from multiple time-points or survival curves were available, we concentrated on the time point with the largest difference between the wildtype and the mutant infection.

Using this count data, we calculated the effect size for each experiment as the log-odds-ratio $= \ln((m_{\text{virulent}}/m_{\text{non-virulent}})/(w_{\text{virulent}}/w_{\text{non-virulent}}))$, where m_{virulent} and w_{virulent} are the number of individuals that died or experienced tissue damage when infected by the mutant and the wildtype strain, respectively, and $m_{\text{non-virulent}}$ and $w_{\text{non-virulent}}$ are the number of individuals that survived or remained unharmed by the infection. Information on the sample size was used to calculate the 95% confidence interval for each effect size and for weighting effect sizes relative to one another (see details below). Where experiments reported a range of sample

sizes, we used the arithmetic mean. Some studies reported only a minimum sample size. In those cases, we used this number. For experiments using *C. elegans*, infections were often carried out on replicate petri dishes in a large number of individuals. In these cases, we used the total number of individual worms used in each treatment group as sample size, and not the number of replica plates.

Additional Analysis: Effect of Pyoverdine on Growth in Mammalian Hosts

Twelve experiments conducted in mammals that were excluded from our main meta-analysis contained data on *in vivo* growth of wildtype and pyoverdine deficient strains. Ten of these came from two publications that also supplied virulence measures meeting our inclusion criteria, while the remaining two came from studies that did not supply any virulence measures (see Table S4). While growth is at best an indirect proxy for virulence, the role of pyoverdine in facilitating in-host growth is an interesting question to ask when considering the *in vivo* role of this molecule. Therefore, we conducted a second, much smaller meta-analysis of these 12 datasets to investigate whether pyoverdine production affects *P. aeruginosa* growth *in vivo*. We calculated the standardized mean differences in growth (bacterial cell counts per gram of tissue or ml of blood) between wild-type and mutant infections for this set of studies (Table S4, Figure S2). This secondary dataset shows a similar pattern to the main meta-analysis shown in **Figure 1**.

Basic Analytical Approach

Analyses were performed in R version 3.3.1 (R Development Core Team, 2016), using functions from packages "meta" (Schwarzer, 2016) and "metaphor" (Viechtbauer, 2010). We used the "metabin" function to transform the count data into the (log-) odds ratio described above. We then weighted these values by the inverse of their respective squared standard errors, and pooled them to obtain a single distribution of effect sizes. We reasoned that the variability of the effect sizes in our dataset probably reflects more than simple sampling error around a single true mean. Rather, we assume that our effect sizes represent a random sample from a larger distribution comprising all possible true effect size estimates. As such, we inferred that a random effects meta-analysis would be more appropriate for our dataset than a fixed effects model (for further discussion, see Borenstein et al., 2009, 2010). In a random effects meta-analysis, we partition the total heterogeneity observed in our dataset (described by the statistic Q) into two constituent parts—within-experiment variation (ε) and between-experiment variation (ζ). The latter component, scaled appropriately to account for the weightings intrinsic to meta-analysis, is quantified as the τ^2 statistic. There are several different algorithms one can use to effect this partitioning of variance. We chose a restricted maximum likelihood (REML) approach. The use of a random model, rather than a simpler fixed model, affects the weights accorded to each constituent effect size, which in turn changes our estimates for pooled means and their associated errors. We further slightly broadened confidence intervals and weakened test statistics using Knapp and Hartung's algorithm (Knapp and Hartung, 2003)—a widely-used and conservative adjustment designed to account for the inherent uncertainty associated with the partitioning of heterogeneity we perform in the course of fitting a random effects model.

We assessed the degree of residual heterogeneity in our dataset using statistics I^2 and H. I^2 estimates the approximate percentage of total variability across experiments that is attributable to unexplained heterogeneity, as opposed to simple sampling error (chance). It is calculated as $100\% \times (Q–df)/Q$, where Q is Cochrane's heterogeneity statistic and df its associated degrees of freedom. H is directly related to I^2 (see equation in Higgins and Thompson, 2002) and reports "excess" heterogeneity as a fold difference compared to the baseline amount of variability we would have expected if the sample were homogenous (Higgins and Thompson, 2002).

Both metrics described above indicated considerable residual heterogeneity in our dataset, so we inferred that, beyond the random- and sampling error, some measurable characteristics of the experiments in our dataset could be contributing, in predictable ways, to the observed heterogeneity of our assembled effect sizes. We therefore extended our basic analysis to take into consideration four potential moderator variables, which we describe below.

Stratification by Moderator Variables

We selected and defined moderator variables on the basis of (a) our *a priori* expectation that they may be important, and (b) the availability of data. The four we investigated were host taxon, infection type, the wildtype strain background, and the expected level of pleiotropy associated with mutations involved. In cases where information was missing for a specific moderator variable, we contacted the authors to obtain additional information. For each moderator, we defined the following relevant subgroups.

Host Organism

Because immune responses and the chemistry of infected tissue/fluid will vary between host taxa, host taxon could reasonably be expected to influence both wild-type levels of virulence and the contribution of siderophores to growth and/or virulence. We first split experiments into broad taxonomic units (mammals, invertebrates, plants), and then classified hosts by genus.

Infection Type

Similarly, different organs or tissue types, even within the same host, could provide different environments and nutrient regimes for bacteria. We classified experiments according to the organ or body region targeted by the infection. Major categories include infections of the host organisms' respiratory tract, digestive system, skin (including burn wounds), and infections that generated a non-localized infection of the body cavity (systemic infection). Experiments that did not fit in any of these categories, such as infections of whole seedlings, were classified as "other infection types."

Wildtype Strain Background

Four different *P. aeruginosa* wildtypes (PAO1, PA14, FRD1, and PAO6049) were used for infection experiments. These are known

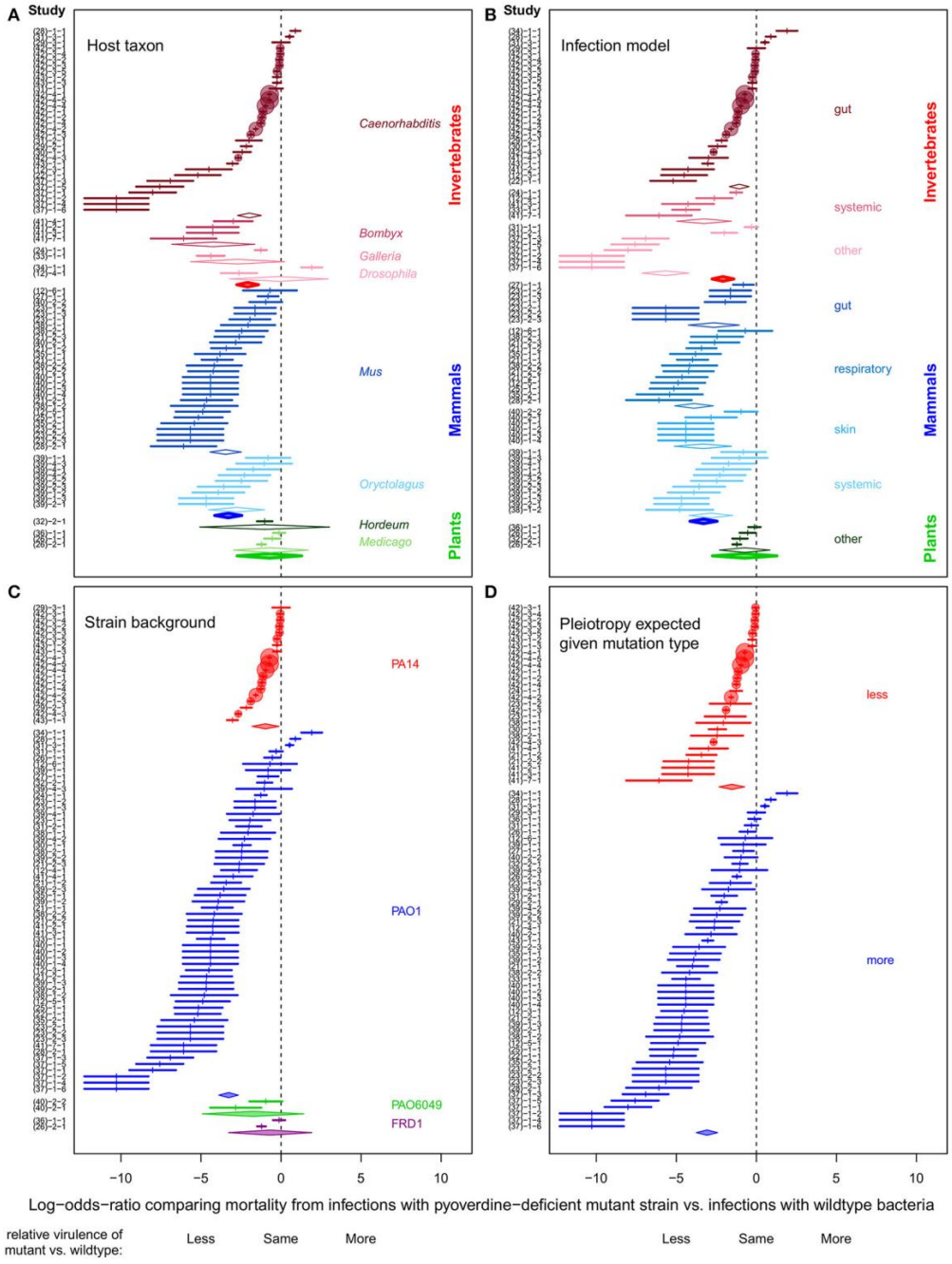

FIGURE 1 | Forest plots depicting the variation in effect size across experiments on pyoverdine as a virulence factor in *P. aeruginosa*. All panels display the same effect sizes originating from the 81 experiments involved in the meta-analysis, but grouped differently according to four moderator variables, which are: **(A)** host taxon; **(B)** infection type; **(C)** wildtype strain background; and **(D)** the likelihood of pleiotropy in the pyoverdine-deficient strain. Effect sizes are given as log-odds-ratio ± 95% confidence interval. Negative and positive effect sizes indicate lower and higher virulence of the pyoverdine-deficient mutant relative to the wildtype, respectively. The scale of the circular markers is proportional to each experiment's relative weighting in the context of a (fixed) meta-analysis. As this weighting is the inverse variance associated with an observed effect, less "noisy" experiments are accorded larger weights. Diamonds represent the mean effect sizes (obtained from meta-regression analysis) for each subgroup of a specific moderator variable. IDs of the individual experiments are listed on the Y-axis (for details, see Table S1 in the Supplemental Material). The numbers in brackets on the Y-axis correspond to the citation number of the corresponding publication.

to differ from one another genetically and phenotypically: for instance, PA14 is generally found to be more virulent than PAO1 across a range of lab models. As background genotype and virulence levels might affect the impact of siderophore mutation on virulence, we included wild-type strain in our analysis. Although it is well established that even standard strains such as PAO1 can substantially differ between labs, there was not enough information available to take such strain-level variation into account.

Likelihood of Pleiotropy

The focal phenotype investigated in this meta-analysis is the production of pyoverdine, the main siderophore of *P. aeruginosa*. Mutants exhibiting reduced or no pyoverdine production can be generated either by deleting a specific pyoverdine-synthesis gene, or through untargeted mutagenesis (e.g., UV light, Hohnadel et al., 1986). The latter mutants are likely to have mutations in other genes unrelated to pyoverdine synthesis. These mutations are typically unknown but could also affect virulence. In principle, even single gene deletions can have pleiotropic effects on the phenotype, via disruption of interactions with other genes. Depending on the locus in question, certain genetic modifications are more likely to have pleiotropy—and so affect the expression of other virulence factors—than others. To account for these complications, we inferred on a case-by-case basis whether the mutation used was likely to only induce a change in (or loss of) pyoverdine production (i.e., pleiotropy less likely) or was likely to induce a change in other phenotypes as well (i.e., pleiotropy more likely). In the biosynthesis of pyoverdine, multiple enzymes are involved in non-ribosomal peptide synthesis (Visca et al., 2007). Two gene clusters, the *pvc* operon and the *pvd* locus, encode proteins involved in the synthesis of the chromophore and peptide moieties, respectively (Stintzi et al., 1999; Visca et al., 2007). In most of these genes, a mutation or deletion leads to a complete loss of pyoverdine production, and most likely does not affect any other trait. Accordingly, we assigned mutants carrying mutations in these genes to the category "pleiotropy less likely." An exception is *pvdQ*, a gene coding for a periplasmic hydrolase, which is required for pyoverdine production, but is also involved in the degradation of N-acyl-homoserine lactone quorum-sensing molecules (Nadal Jimenez et al., 2010). Strains with deletions in this gene were therefore assigned to the category "pleiotropy more likely." Other strains falling into this category included: (i) mutants where the key regulator of pyoverdine synthesis, PvdS, was deleted, leading to deficiencies in toxin and protease production, in addition to a complete loss of pyoverdine production (Beare et al., 2003); (ii) strains that carry a deletion in a central metabolic gene and only coincidentally show no (or strongly reduced) pyoverdine production; (iii) double mutants that carry deletions in both the pyoverdine and the pyochelin synthesis pathway (pyochelin is the secondary siderophore of *P. aeruginosa*, Braud et al., 2009); and (iv) pyoverdine mutants created via non-targeted (e.g., UV) mutagenesis.

Using the stratification described above, we focused on estimating mean effect sizes and their associated confidence intervals within all subgroups represented in our full dataset (i.e., the diamonds in **Figure 1**). To this end, we fitted a series of four univariate models, one for each moderator. Each model was a random-effects meta-analysis (as above) but we constrained the level of between-experiment heterogeneity (τ^2) to be common across all subgroups (i.e., each factor level of the moderator under examination).

Comparison of Moderator Variables' Relative Influence

To better understand the relative importance of the four moderator variables described above, we focused on a reduced "core" dataset, for which we excluded experiments belonging to rare or poorly characterized subgroups to generate a smaller but more homogenous dataset. We excluded experiments involving plants and/or undefined wildtype strains ($n = 6$), experiments reporting tissue damage as a measure of virulence ($n = 12$), and experiments where the hosts were likely not colonized by bacteria but died from exposure to bacterial toxins ($n = 8$). This resulted in a core dataset comprising 55 experiments.

Using this dataset, we then fitted meta-regression models that simultaneously considered the contributions of multiple moderator factors. Our models were able to estimate moderators' additive effects only, because even in this "core" dataset, with its comparatively better data coverage across the remaining subgroups, the distribution of data across different combinations of factor levels was still too patchy to permit a proper investigation of moderators' interactive effects. Moderators' alterations of the expected (i.e., baseline) effect size could be quantified as coefficients, which could, when standardized as *t*-statistics, be tested for significant differences from zero. In addition, we could test whether, collectively, the inclusion of moderators in our meta-analysis model significantly reduced the residual heterogeneity relative to a situation with no moderators.

To estimate what share of the residual heterogeneity in our dataset could be individually attributable to each of the respective moderators, we performed a series of likelihood ratio tests comparing, in each case, a full model including all four moderators, against a reduced model that excluded one of the moderators. Variance component estimation in these models used maximum likelihood instead of REML because nested REML models cannot be compared in this way. From each pairwise comparison, we obtained a pseudo-R^2 value, which reflects the difference in τ^2 (between-experiment heterogeneity) between the two models, scaled by the τ^2 of the simpler model.

Potential Within-Study Bias

The most likely source of potential directional bias within studies is the genotype of the siderophore mutant used. One unpublished dataset was excluded partly because the siderophore mutant used carried a mutation in a locus that is not essential for siderophore production (Table S3), and so experiments using this mutant may be biased toward finding no effect of mutation on virulence. Sixteen included datasets used *pvdS* mutants; because *pvdS* positively regulates the expression of other virulence factors as well as siderophores, experiments using this mutant may be biased toward finding an effect of mutation on virulence. Similarly, three included datasets used a UV-generated

mutant, PAO6609 (PAO9) which carries mutations in several loci affecting growth or virulence (F. Harrison, A. McNally, A. Da Silva & S. P. Diggle, unpublished data). Because we included likelihood of pleiotropy as a moderator variable in our analysis, we should be able to partition out between-study variance caused by the use of mutants that risk a bias toward positive results.

Testing for Signs of Publication Bias

To test for putative publication bias in our dataset, we compared effect sizes against their respective standard errors, the idea being that if there is no bias, there should be no link between the magnitude of the result from a given experiment, and the "noisiness" or uncertainty of that particular result. If there is bias, we could find an overrepresentation of noisier experiments reporting higher magnitude results. Using the "metabias" function of the R package "meta," we performed both (weighted) linear regressions and rank correlations to test for this pattern (Begg and Mazumdar, 1994; Egger et al., 1997).

RESULTS

Literature Search and Study Characteristics

We searched the literature for papers featuring infections of whole live host organisms with *P. aeruginosa* strains known to vary in pyoverdine phenotype. Following a set of inclusion/exclusion rules (see materials and methods for details), we were able to include data from a total of 81 experiments from 24 original papers in our meta-analysis (**Table 1**; see also Figure S1 and Tables S1, S2 in the Supplemental Material). These experiments featured a range of host organisms, including mammals (mice and rabbits, $n = 37$), the nematode *Caenorhabditis elegans* ($n = 32$), insects (fruit fly, silk worm and wax worm, $n = 8$) and plants (wheat and alfalfa, $n = 4$). Experiments further differed in the way infections were established and in the organs targeted. The most common infection types were gut ($n = 34$), systemic ($n = 16$), respiratory ($n = 13$) and skin infections ($n = 6$), but we also included some other types of infections ($n = 12$). Each experiment compared infections with a control *P. aeruginosa* strain (which produced wildtype levels of pyoverdine) to infections with a mutant strain defective for pyoverdine production. The most common control strains used were PAO1 ($n = 58$) and PA14 ($n = 19$), which are both well-characterized clinical isolates. However, some experiments used less well-characterized wildtype strains, such as FRD1 ($n = 2$) and PAO6049 ($n = 2$). Twenty-eight experiments used mutant strains with clean deletions or transposon Tn5 insertions in genes encoding the pyoverdine biosynthesis pathway. In these cases, pleiotropic effects are expected to be relatively low—i.e., presumably only pyoverdine production was affected. The other 53 experiments used mutants where pleiotropic effects were likely or even certain. For example, some mutant strains carried mutations in *pvdS*, which encodes the main regulator of pyoverdine synthesis that also regulates the production of toxins and proteases (Ochsner et al., 1996; Wilderman et al., 2001). Others carried mutations in *pvdQ*, encoding an enzyme known to degrade quorum-sensing molecules in addition to its role in pyoverdine biosynthesis (Nadal Jimenez et al., 2010).

Mean Effects across and Within Subgroups

We combined data from the set of experiments described above in a meta-analysis to determine the extent to which pyoverdine's effect on virulence varied across four moderator variables: (i) host taxa, (ii) tissue types, (iii) pathogen wildtype background, and (iv) pyoverdine-mutation type. To obtain a comparable measure of virulence across experiments, we extracted in each instance the number of cases where a given infection type did or did not have a virulent outcome (i.e., dead vs. alive, or with vs. without symptoms) for both the mutant (m) and the wildtype (w) strain for each experiment (see materials and methods for details). We then took as our effect size the log-odds-ratio, i.e., $\ln((m_{\text{virulent}}/m_{\text{non-virulent}})/(w_{\text{virulent}}/w_{\text{non-virulent}}))$ (see Table S2 in the Supplemental Material), a commonly-used measure especially suitable for binary response variables like survival (Szumilas, 2010).

Consistent with the theoretical prediction that host-pathogen interactions and host ecology are important modulators of virulence, we found considerable variation in the effect sizes across experiments and subgroups of all moderators (**Figure 1**). Pyoverdine-deficient mutants showed substantially reduced virulence in invertebrate and mammalian hosts, whereas there was little evidence for such an effect in plants (**Figure 1A**). Overall, evidence for pyoverdine being an important virulence factor was weak for taxa with a low number of experiments (i.e., for plants, and the insect models *Drosophila melanogaster* and *Galleria mellonella*). We found that pyoverdine-deficient mutants exhibited reduced virulence in all organs and tissues tested, with the exception of plants (**Figure 1B**). Comparing the effect sizes across wildtype strain backgrounds, we see that pyoverdine deficiency reduced virulence in experiments featuring the well-characterized PA14 and PAO1 strains (**Figure 1C**) whereas the reduction was less pronounced in experiments with less well-characterized wildtype strains. This could be due to sampling error (only a few experiments used these strains) or it may be that these strains really behave differently from PA14 and PAO1. Finally, we observed that the nature of the pyoverdine-deficiency mutation matters (**Figure 1D**). Infections with strains carrying well-defined mutations known to exclusively (or at least primarily) affect pyoverdine production showed a relatively consistent reduction in virulence. Conversely, where mutants were poorly-defined, or carried mutations likely to affect other traits beyond pyoverdine, here the virulence pattern was much more variable, with both reduced and increased virulence relative to wildtype infections (**Figure 1D**). We posit that at least some of the differences in observed virulence between these mutants and their wildtype counterparts was likely due to pleiotropic differences in phenotypes unrelated to pyoverdine.

Relative Importance of Moderator Variables

Figure 1 highlights that we are dealing with an extremely heterogeneous dataset (a random meta-analysis of the full dataset

without moderators yielded heterogeneity measures $I^2 = 97.92\%$ (97.16–98.48) and $H = 6.93$ (5.93–8.10), where values in brackets indicate the 95% confidence limits associated with each estimate). Much of the variation we observe is probably due to other factors beyond those explored in **Figure 1**. The issue is that (a) we do not know what all these additional factors might be, and (b) the probably patchy distribution of experiments across the levels and ranges of these other factors would leave us with limited power to test for their effects. Accordingly, we decided to focus our attention on quantifying the impact of the four previously described moderators by using a more homogenous core dataset ($n = 55$), where rare and poorly characterized subgroups were removed. Specifically, we excluded experiments involving plants and/or undefined wildtype strains ($n = 6$), experiments reporting tissue damage as a measure of virulence ($n = 12$), and experiments where the hosts were likely not directly colonized by bacteria but died from exposure to bacterial toxins ($n = 8$). This leaves us with a core dataset comprising only those experiments where animal host models were infected with strains from well-defined PA14 or PA01 wildtype background, and survival vs. death was used as a virulence endpoint.

Using this restricted dataset, we performed a series of meta-regression models to test for significant differences between subgroups of our moderator factors, and we also estimated the share of total variance in effect sizes that is explained by each moderator variable (**Figure 2**). These models revealed that infection type is the variable that explains the largest share of total variance (25.4%). For instance, in systemic infection models the pyoverdine-defective mutants showed strongly reduced virulence compared to the wild-type, whereas this difference was less pronounced in gut infections. Host taxon explained only 8.2% of the total variance in effect sizes, and there was no apparent difference in the mean effect size among invertebrate vs. mammalian host models. Finally, the wildtype strain background and the likelihood of pleiotropy in the mutant strain both explained less than 1% of the overall effect size variation, and accordingly, there were no apparent differences between subgroups (**Figure 2**). This was interesting, because we predicted *a priori* that mutations with pleiotropic effects on other virulence factors could introduce within-study bias toward a greater effect of siderophore loss on virulence. Note that even with the inclusion of these moderator factors in the model, substantial heterogeneity remained in our restricted data set [$I^2 = 96.15\%$ (94.23–97.43), $H = 5.10$ (4.16–6.24)].

Publication Bias

In any field, there is a risk that studies with negative or unanticipated results may be less likely to get published (e.g., in our case, pyoverdine-deficient mutants showing no change or increased levels of virulence, Dwan et al., 2008). Especially when negative or unanticipated results are obtained from experiments featuring low sample sizes (and thus high uncertainty), the scientists responsible may be less inclined to trust their results, and consequently opt not to publish them. This pattern could result in a publication bias, and an overestimation of the effect size. To test whether such a publication bias exists in our dataset, we plotted the effect size of each experiment against its (inverted) standard error (**Figure 3**). If there is no publication bias, we would expect to see an inverted funnel, with effect sizes more or less evenly distributed around the mean effect size, irrespective of the uncertainty associated with each estimate (i.e., position on the y-axis). Instead, we observed a bias in our dataset, with many lower-certainty experiments that show strongly negative effect sizes (i.e., supporting the hypothesis that pyoverdine is important for virulence; **Figure 3**) but a concomitant paucity of lower-certainty experiments that show weakly negative, zero or positive effect sizes (i.e., not supporting the hypothesis).

DISCUSSION

What Have We Learned from this Meta-Analysis

Our meta-analysis reveals that pyoverdine-deficient strains of the opportunistic pathogen *P. aeruginosa* typically showed reduced virulence across a wide range of host species and bacterial genotypes. This confirms that iron limitation is a unifying characteristic of the host environment, making siderophores an important factor for pathogen establishment and growth within the host (Parrow et al., 2013; Becker and Skaar, 2014). However, we also saw that the extent to which pyoverdine deficiency reduced virulence varied considerably, and was quite modest in many instances. Pyoverdine-deficient mutant strains were typically more benign, owing to a reduced capacity for *in vivo* growth and/or a reduced capacity for inflicting damage on their host. Nonetheless, these mutants were typically still able to establish a successful infection, and, in many cases, could still kill their host (Romanowski et al., 2011; Kirienko et al., 2013; Ross-Gillespie et al., 2014; Lopez-Medina et al., 2015). These results support ecological theory predicting that the effect of a certain phenotype (i.e., producing pyoverdine in our case) should vary in response to the environment (i.e., the host and infection context, Lambrechts et al., 2006).

Our findings have direct consequences for any therapeutic approaches targeting this particular virulence factor. Because pyoverdine seems to be generally involved with virulence, treatments inhibiting pyoverdine production could have wide applicability and be effective against different types of infections across a wide host range. However, given the variation observed and pyoverdine's generally modest effect on virulence, the clinical impact of such treatments would likely vary across infection contexts, and be limited to attenuating rather than curing the infection. This would mean that for *P. aeruginosa* infections, at least, therapies targeting siderophore production could be helpful but should probably still be accompanied by other therapeutic measures, and applied for instance in combination with an antibiotic treatment (Banin et al., 2008). Certainly promising is that pyoverdine seems to have a more consistent (**Figure 1A**) and more prominent (**Figure 2** although not significant) effect in mammalian compared to invertebrate hosts. From this observation, one could infer that pyoverdine may have potential as a target for infection control in humans.

Our work demonstrates how meta-analyses can be used to quantitatively synthesize data from different experiments

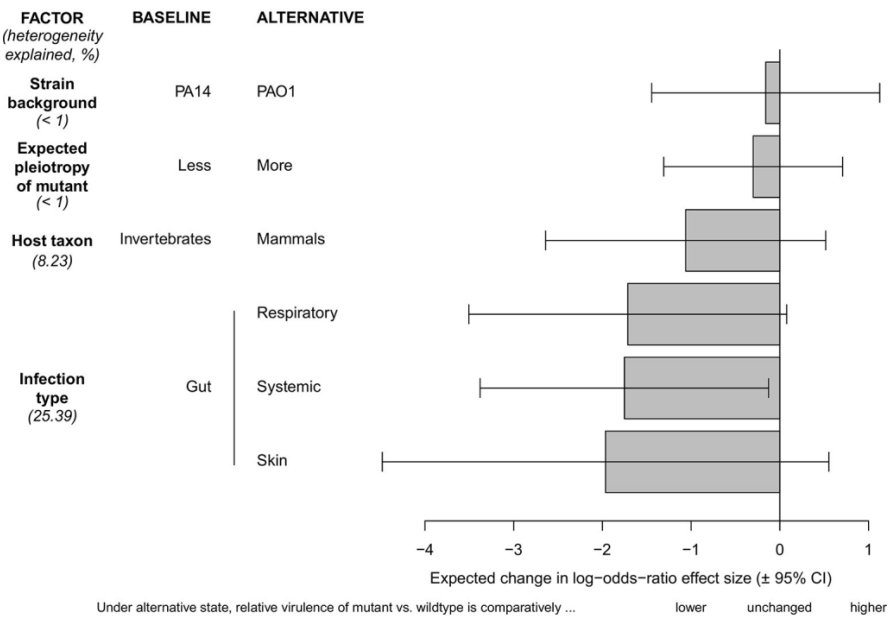

FIGURE 2 | Test for differences between subgroups of moderator variables with regard to the effect sizes for pyoverdine as a virulence factor in *P. aeruginosa*. Our baseline condition for all comparisons is the following: gut infections in invertebrate hosts, using the *P. aeruginosa* wildtype strain PA14 vs. a pyoverdine-deficient PA14 mutant with a low expected level of pleiotropy. The effect size for this baseline scenario is set to zero. All other scenarios had more extreme (negative) effect sizes, and are therefore scaled relative to this baseline condition. Comparisons reveal that virulence in pyoverdine-deficient strains was significantly more reduced in systemic compared to gut infections, and that most effect size variation is explained by the infection type. There were no significant effect size differences between any of the other subgroups. Bars show the difference in log odds-ratio (± 95% confidence interval) between the baseline and any of the alternate conditions. Values given in brackets indicate percentage of effect size heterogeneity explained by a specific moderator.

carried out at different times by different researchers using different designs. Such an analytical approach goes beyond a classical review, where patterns are typically summarized in a qualitative manner. For instance, a recent study proposed that three different virulence factors (pyocyanin, protease, swarming) of *P. aeruginosa* are host-specific in their effects (Dubern et al., 2015). Here we use a meta-analytic approach to quantitatively derive estimates of the overall virulence potential of a given bacterial trait and investigate variables that affect infection outcomes. We assert that such quantitative comparisons are essential to identify those virulence factors that hold greatest promise as targets for effective broad-spectrum anti-virulence therapies.

Our finding that effect sizes vary considerably across our assembled experiments provides a different perspective compared to that which one would obtain from a cursory reading of the literature. For instance, the first study investigating pyoverdine in the context of an experimental infection model (Meyer et al., 1996) reported that pyoverdine is essential for virulence. Although this experiment and its message have been widely cited (including by ourselves), it may no longer be the strongest representative of the accumulated body of research on this topic. As we see in **Figure 1**, the effect size it reports is associated with a high uncertainty due to a comparatively low sample size. Moreover, the observed effect cannot unambiguously be attributed to pyoverdine because an undefined UV-mutagenized mutant was used. We highlight this example not to criticize it, but rather because it serves to demonstrate why drawing inferences from (appropriately weighted) aggregations of all available evidence is preferable to focusing solely on the results of a single study.

Areas of Concern

Our meta-analytic approach not only provides information on the overall importance of pyoverdine for *P. aeruginosa* virulence, but it also allows us to identify specific gaps in our knowledge. For example, let us consider which types of studies were conspicuously absent from our dataset. First, most experiments in our dataset employed acute infection models, even though *P. aeruginosa* is well known for its persistent, hard-to-treat chronic infections. This raises the question to what extent insights on the roles of virulence factors important in acute infections can be transferred to chronic infections. In the case of pyoverdine, we know that in chronically-infected cystic fibrosis airways, pyoverdine production is often selected against (Wiehlmann et al., 2007; Jiricny et al., 2014; Andersen et al., 2015). Although the selective pressure driving this evolutionary loss is still under debate (current explanations include pyoverdine disuse, competitive strain interactions and/or a switch to alternative iron-uptake systems, Marvig et al., 2014;

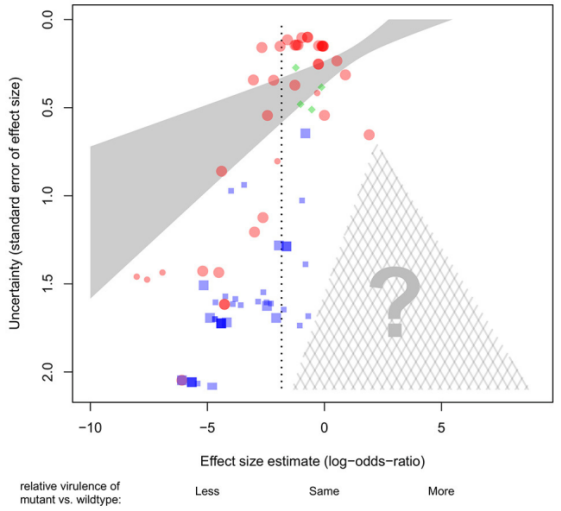

FIGURE 3 | Association between effect sizes and their standard errors across 81 experiments examining the role of pyoverdine production for virulence in *P. aeruginosa*. In the absence of bias, we should see an inverted funnel-shaped cloud of points, more or less symmetrically distributed around the mean effect size (vertical dotted line). Instead, we see an over-representation of low-certainty experiments associated with strong (negative) effect sizes. This suggests a significant publication bias: experiments with low-certainty and weak or contrary effects presumably do exist, but are under-represented here (note the absence of data points in the cross-hatched triangle). Effect sizes are given as log-odds-ratio. Each symbol represents a single experiment. Symbol colors and shapes stand for different host organisms (red circles, invertebrates; blue squares, mammals; green diamonds, plants). Large symbols denote the experiments included in the core dataset. The solid shaded area represents the 95% confidence interval for the weighted linear regression using the complete dataset. Note that due to the stronger weights accorded to high certainty experiments (i.e., the points toward the top of the plot), many of the lower-weighted (higher-uncertainty) points toward the bottom of the plot lie quite far from the regression line and also outside the confidence interval.

Andersen et al., 2015; Kümmerli, 2015), this example illustrates that the role of pyoverdine might differ in acute vs. chronic infections.

Second, our comparative work shows that experiments were predominantly carried out with the well-characterized strains PAO1 and PA14. While these strains were initially isolated from clinical settings, they have subsequently undergone evolution in the laboratory environment (Bragonzi et al., 2009; Klockgether et al., 2011; Frydenlund Michelsen et al., 2015), and might now substantially differ from the clinical strains actually causing acute infections in hospitals. Therefore, while we found no overall differences between the lab strains used in our data set, we argue that it would still be useful to carry out additional studies on a range of clinical isolates to be able to make firm conclusions on the general role of pyoverdine as a virulence factor.

Finally, our data analysis revealed that low-certainty studies showing no or small effects of pyoverdine on virulence were under-represented in our data set, which points toward a systematic publication bias. It remains to be seen whether such biases are common with regard to research on virulence factors, and whether they result in a general overestimation of the effect these factors have on host survival or tissue damage. With regard to pyoverdine, further studies are clearly needed to obtain a more accurate estimate of the true effect size.

In addition to these issues of data availability, all meta-analyses unavoidably involve intrinsic assumptions and subjective decisions that can further influence the resulting outputs. For instance, although we have used the standard log-odds-ratio as our common metric, related metrics like risk ratios, while typically highly correlated, can sometimes produce different patterns in a meta-analysis. In the present case, however, using risk ratios instead does not qualitatively affect the patterns we observed nor alter our conclusions. Furthermore, to facilitate calculation of a log-odds ratio in cases where zero counts appear as denominators, we have in this study adopted the common, yet ultimately arbitrary, convention of replacing these zero denominators with 0.5. Had we used a different value as a substitute, say 0.1 or 0.9, the estimates from our resulting models would have been different—at least quantitatively. A more fundamental issue is that when estimating population-level statistics across a collection of experiments, we typically assume that we are comparing like with like. Here, we have intentionally brought together a very diverse set of studies, and even though we have translated their individual effect sizes into a common metric and also stratified them by some of their major defining characteristics, their individual effect sizes nonetheless remain highly heterogeneous. In effect, we are knowingly combining apples and pears, because we think that the resulting fruit salad is still something that is worth taking a look at. Alternative or additional ways of slicing up the data could yield models with lower residual heterogeneity, but then poor data coverage in combinations of subcategories could limit the accuracy of any parameter estimates we want to extract from such models. In light of these issues, we advise readers to focus on the overall patterns our models reveal, rather than the specific values of the estimates they generate.

Guidelines for Future Studies

While our study demonstrates the strength of quantitative comparative approaches, it is important to realize that extracting effect sizes is one of the biggest challenges in any meta-analysis. This challenge was particularly evident for the experiments we found, which profoundly varied in the way data was collected and reported. As a consequence, we had to exclude many studies because they used measures of virulence that were only reported by a minority of studies, or because their reporting of results was unclear (for a selected list of examples, see Table S3 in the Supplemental Material). To amend this issue for future studies, we would like to first highlight the problems we encountered and then provide general guidelines of how data reporting could be improved and standardized. One main problem we experienced was incomplete data reporting (i.e., mean treatment values, absolute values and/or sample size was not reported), which prevents the calculation of effect sizes and uncertainty measures. Another important issue was that different studies measured virulence using very different metrics. Some measured virulence

at the tissue level (i.e., the extent of damage inflicted), while others focused on the whole host organism. Others focused on the dynamics of the bacteria themselves, taking this as a proxy for the eventual damage to the host. There were both quantitative measures (e.g., extent of damage), and qualitative measures (e.g., assignments to arbitrary categories of virulence). Survival data was sometimes presented as a timecourse, sometimes as an endpoint; sometimes as raw counts, sometimes as proportions. In most cases, the time scales over which survival was assessed were fairly arbitrary. Compiling such diverse measures of virulence is not simply time consuming, but it also generates extra sources of heterogeneity in the dataset, which might interfere with the basic assumptions of meta-analytical models (Borenstein et al., 2009, 2010).

How can these problems be prevented in future studies? We propose the following. (a) Whenever possible, time-to-event data (e.g., death, organ failure, etc.) should be recorded in a form that preserves both the outcome and the times to event per subject. (b) The number of replicates used (hosts) and a measure of variance among replicates must be provided to be able to calculate a confidence estimate for the experiment. (c) If data are scaled in some way (e.g., relative to a reference strain), the absolute values should still be reported, because these are crucial for the calculation of effect sizes. Finally, (d) studies leading to unexpected or negative results (e.g., no difference in virulence between a wildtype and a mutant) should still be published, as they are needed to estimate a true and unbiased effect size. In summary, all findings, irrespective of their magnitude or polarity, should be presented "as raw as possible" (e.g., in Supplementary files or deposited in online data archives). This will make comparisons across studies much easier and will provide a useful resource for future meta-analytic studies.

CONCLUSIONS

Currently, bacterial traits are subject to a binary categorisation whereby some are labeled as virulence factors while others are not. We demonstrate that traits' effects on virulence are anything but binary. Rather, they strongly depend on the infection context. Our study affirms meta-analysis as a powerful tool to quantitatively estimate the overall effect of a specific virulence factor and to compare its general importance in infections across different bacterial strains, hosts, and host organs. Such quantitative comparisons provide us with a more complete picture on the relative importance of specific virulence factors. Such knowledge is especially valuable for opportunistic pathogens, which have a wide range of virulence factors at their disposal, and infect a broad range of host organisms (Kurz et al., 2003; He et al., 2004; Calderone and Fonzi, 2001). Meta-analytical comparisons could thus inform us on which traits would be best suited as targets for anti-virulence therapies. Ideal traits would be those with high effect sizes and general importance across pathogen and host organisms.

AUTHOR CONTRIBUTIONS

EG, FH, RK, and AR-G conceived the study; EG conducted the literature search and compiled the data set; AR-G conducted statistical analysis; EG, FH, RK, and AR-G interpreted the data and wrote the paper.

FUNDING

Forschungskredit Candoc of the University of Zurich (EG). Swiss National Science Foundation PP00P3-139164 (AR-G, EG, and RK). Novartis Foundation for Medical and Biological Research (AR-G and RK). University of Warwick (FH).

ACKNOWLEDGMENTS

We thank Natasha Kirienko and Kendra Rumbaugh for sharing raw and unpublished data and Richard Allen, Steve Diggle, Sinead English, James Gurney, Steven Higgins, and Shinichi Nakagawa for comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01952/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.2 Supporting Material

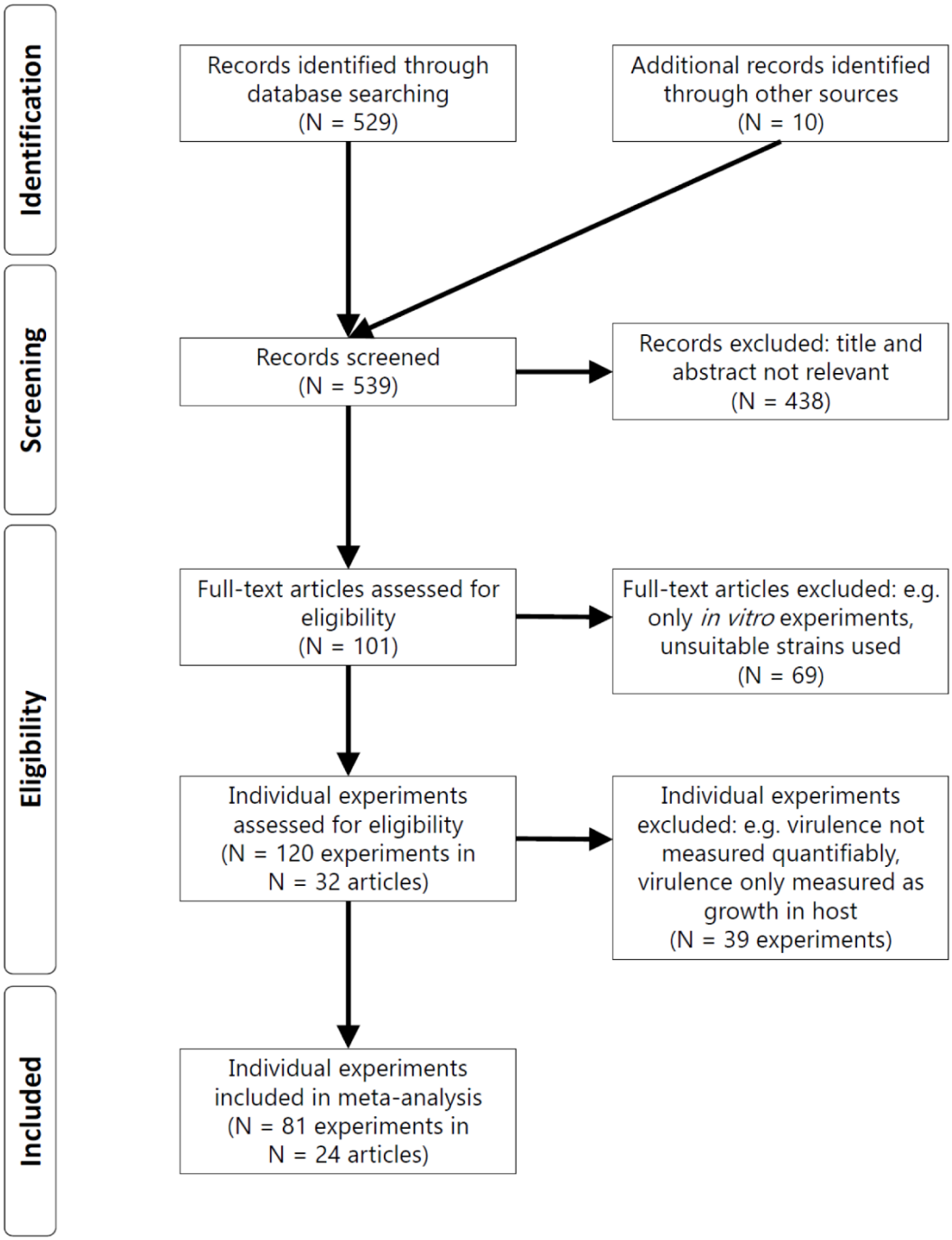

Fig. S1 Flow diagram (PRISMA format) of the screening and selection process for studies investigating the association between pyoverdine production and virulence in *P. aeruginosa*.

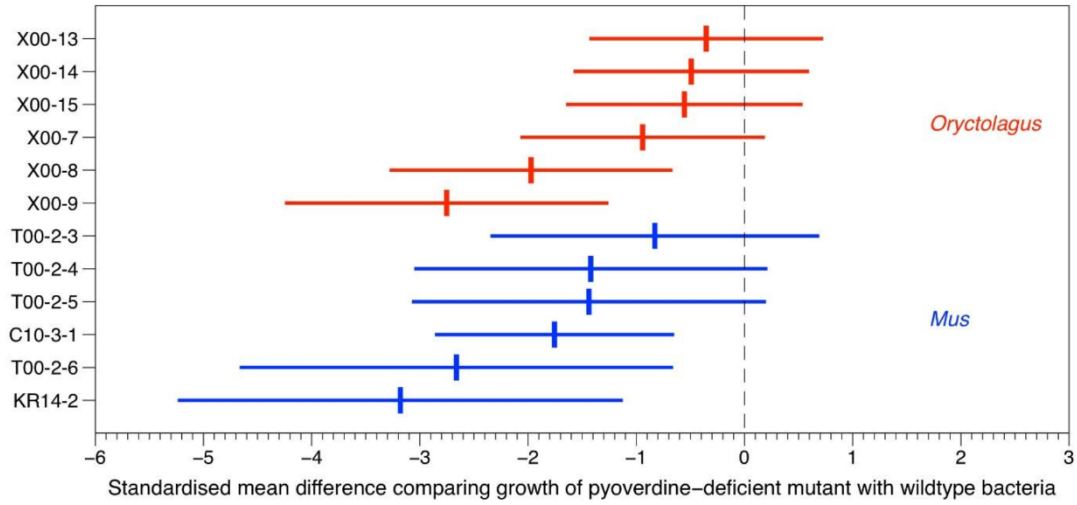

Fig. S2 Forest plot depicting the variation in effect size across experiments on the effect of pyoverdine on the growth of *P. aeruginosa* in mammalian hosts. Effect sizes are given as standardized mean difference ± 95% confidence interval and are grouped by host genus. Negative and positive effect sizes indicate lower and higher *in vivo* growth of the pyoverdine-deficient mutant relative to the wildtype, respectively. IDs of the individual experiments are listed on the Y-axis (for details, see Table S4 in the supplemental material).

6 PROJECT 2 – Virulence Evolution

"I would rather be an opportunist and float,

than go to the bottom with my principles around my neck."

~Stanley Baldwin, British politician (1867-1947)

6.1 Drivers of virulence evolution in an opportunistic bacterial pathogen

Drivers of virulence evolution in an opportunistic bacterial pathogen

Elisa T. Granato[1], Christoph Ziegenhain[2], Rolf Kümmerli[1]

[1]Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland.

[2]Department Biology II, Ludwig-Maximilians-University, Munich, Germany.

ABSTRACT

Bacterial opportunistic pathogens are feared for their difficult-to-treat nosocomial infections and for causing morbidity in immunocompromised patients. Here, we study how such a versatile opportunist, *Pseudomonas aeruginosa*, adapts to conditions inside and outside its model host *Caenorhabditis elegans*, and use phenotypic and genotypic screens to identify the mechanistic basis of virulence evolution. We found that virulence dramatically dropped in unstructured environments both in the presence and absence of the host, but remained unchanged in spatially structured environments. The observed virulence decline was driven by two interacting forces: accidental effects, where bacteria became deficient in traits not needed outside the host; and social effects, where mutants that lost the ability to produce shareable virulence factors displaced the wildtype through social exploitation. Our study identifies key drivers of virulence evolution in an opportunistic pathogen, and indicates that disrupting spatial structure in chronic infections could steer pathogen evolution towards lower virulence.

INTRODUCTION

Bacterial opportunistic pathogens are a major cause of morbidity and mortality in immunocompromised humans (e.g. cancer, AIDS, cystic fibrosis patients) [1–3]. Despite their socio-economic relevance for our society [4], we know little about how these opportunists adapt to the variable environments they occupy, and how adaptation affects virulence, and thus damage to the host [5]. Part of the complication is that opportunistic pathogens can adapt both to the external abiotic and the host environment, and it is unclear how selection in the two environments affects pathogenicity [6].

To address this issue, we studied the evolution of virulence in an opportunistic human pathogen, the bacterium *Pseudomonas aeruginosa*, infecting its model host *Caenorhabditis elegans* [7,8]. We independently manipulated key aspects of the abiotic and biotic environment in order to disentangle the role of three commonly proposed drivers of virulence evolution. The first driver is the abiotic non-host environment, which could potentially be the sole factor guiding bacterial evolution, such that any effect we subsequently see in the host is purely accidental [6]. The second driver involves social interactions between pathogens, ranging from cooperation to competition [9–13]. Social interactions matter because they determine the extent to which pathogens compete for resources, which feeds back on their ability to colonize and harm the host. Finally, the third driver is the host environment itself (e.g. immune factors, host physiology) to which pathogens may adapt [14–17].

We let *P. aeruginosa* PAO1 wildtype bacteria evolve for approximately 200 generations in four different environments in eight-fold replication (Fig. 1A). In order to disentangle environmental from host virulence drivers, we cultured bacteria with or without hosts. Furthermore, we let bacteria either evolve in uniform liquid or spatially structured solid medium to manipulate the relative importance of competitive versus cooperative

interactions between pathogens. Theory predicts that increased spatial structure induces a shift from competitive to cooperative interactions because it limits individual dispersal, and thus promotes local interactions among clone-mates with aligned interests [9–12]. This is of special relevance for bacteria because their ability to grow often depends on the secretion of shareable public goods, such as siderophores, secreted enzymes and toxins [18], which are termed virulence factors when expressed within the host [19]. The shareability of these molecules in spatially unstructured environments can promote the evolution of cheater mutants, which stop molecule production, yet still exploit the public goods produced by others [20]. The evolutionary spread of these cheater mutants is supposed to reduce both pathogen growth and the damage inflicted on the host [21–25]. Importantly, our setup allows for the *de novo* evolution of clones with altered levels of virulence factor production, in contrast to other studies where social exploitation is commonly explored with previously constructed clones carrying targeted deletions in biosynthesis pathways.

RESULTS

Prior to experimental evolution, we found that the ancestral wildtype was highly virulent by killing 76.2% and 83.9% of all host individuals within 24 hours in liquid and on solid media, respectively. This pattern changed during evolution in unstructured environments, where virulence dropped by 32.3% and 44.7% for populations that evolved with and without hosts, respectively (Fig. 1B+C, Fig. S1). Conversely, virulence remained high in structured environments. Overall, there was a significant effect of spatial structure on virulence evolution (linear mixed model: $df_{structure} = 24.7$, $t_{structure} = -2.11$, $p_{structure} = 0.045$), while host presence did not seem to matter ($df_{host} = 18.6$, $t_{host} = 0.86$, $p_{host} = 0.40$).

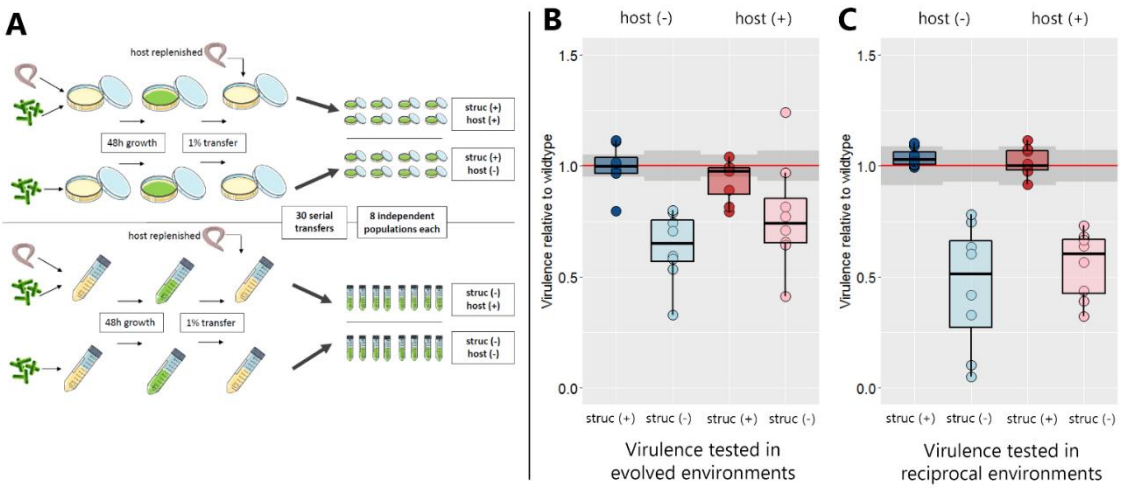

Fig. 1. Virulence decreased during evolution in unstructured environments. (**A**) Experimental design: P. aeruginosa PAO1 bacteria were serially transferred 30 times in four different environments (in 8-fold replication). These environments were either spatially structured ("struc +") or unstructured ("struc —"), and either contained ("host +") or did not contain ("host — ") C. elegans nematodes for the bacteria to infect. Subsequently, the evolved populations were tested for their virulence towards the nematode under two different conditions: (**B**) In the environment the populations evolved in (i.e. populations that evolved on agar plates tested on agar plates, populations that evolved in liquid culture tested in liquid culture); and (**C**) in the reciprocal environment as a control (populations that evolved on agar plates tested in liquid culture, populations that evolved in liquid tested on agar plates). Both assays revealed that virulence significantly decreased during evolution in unstructured environments (Wilcoxon rank-sum test, $p < 0.05$; see Table S1). Virulence was quantified as percent nematodes killed at 24 h post infection, scaled to the ancestral wildtype. Individual dots represent mean virulence of evolved populations. The red line represents the average wildtype virulence level in the respective assay, with shaded areas denoting the 95% confidence intervals.

One way to interpret the absence of a host effect is that evolution of reduced virulence is primarily accidental, either driven by abiotic adaptions to liquid medium, or by selection for cheaters outside the host.

To explore whether altered virulence levels can be explained by changes in the production of publically shareable virulence factors, we quantified the phenotypes of 640 evolved clones for their ability to produce: (i) pyoverdine, required for iron-scavenging [26]; (ii) pyocyanin, a broad-spectrum toxin [27]; and (iii) proteases to digest extracellular proteins [28]. We further quantified the pathogen's ability to attach to surfaces, the early stage of biofilm formation, another social trait typically involved with virulence [29].

Our phenotype screens revealed significant changes in the production of all four virulence factors (Fig. 2). For pyoverdine, we observed a significant decrease in pyoverdine production in unstructured environments without hosts, with many clones (44.4%) having completely lost the ability to produce pyoverdine (Fig. 2A). Since our media was iron-limited, impeding the growth of pyoverdine non-producers, these mutants likely represent cheaters, exploiting the pyoverdine secreted by producers. In all other environments, we found a significant increase in pyoverdine production (Bayesian generalized linear mixed model, BGLMM: $p_{host:structure} = 0.027$).

Notably, in unstructured environments in presence of the host, pyoverdine non-producers did evolve but were not able to spread to such a high degree as in the same environment without the host. This can likely be attributed to the spatial structuring present within individual hosts, which acts to limit the indirect fitness benefits available to cheaters.

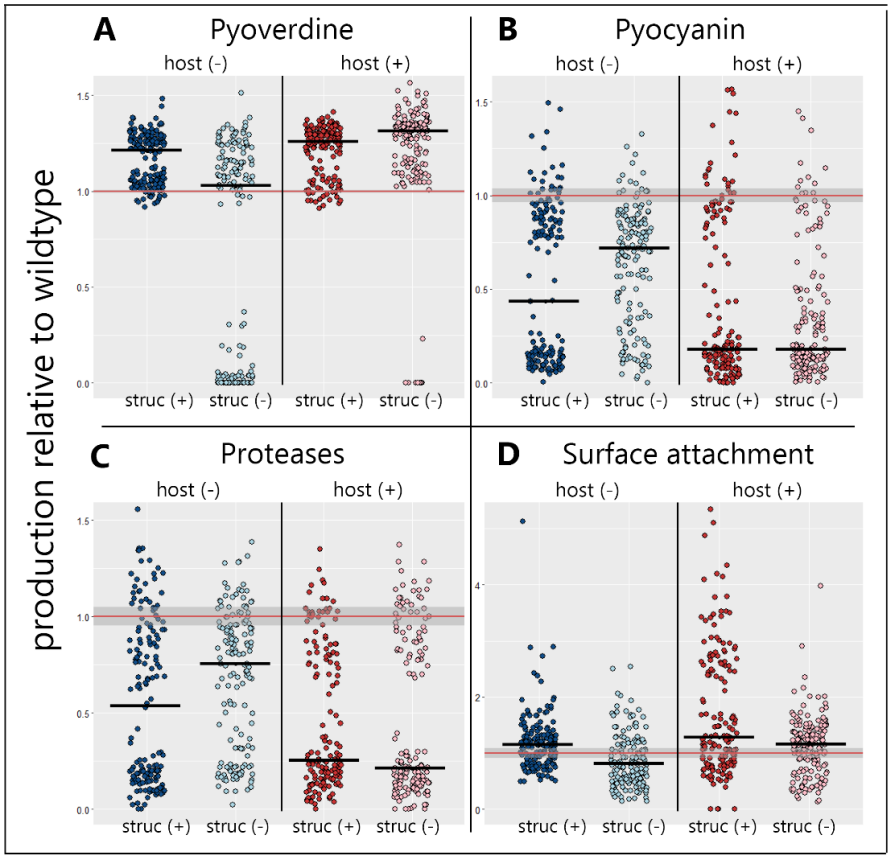

Fig. 2. Selection promoted shifts in virulence factor production during experimental evolution. The production levels of four important virulence factors were determined for 640 evolved *P. aeruginosa* clones (20 clones per evolved line), and compared to the ancestral wildtype (mean ± 95 % confidence intervals indicated as red lines and shaded areas, respectively). (**A**) The production of the siderophore pyoverdine significantly decreased in the host-free unstructured environment, but significantly increased in all other environments. (**B**) The production of the toxin pyocyanin significantly decreased in all environments, but more so in the environments with the host. (**C**) The production of proteases also significantly decreased in all environments, with a sharper decline in environments with the host. (**D**) The clones' ability to attach to surfaces significantly decreased in the unstructured host-free environment, but significantly increased in all other environments. host (—) = host was absent during evolution; host (+) = host was present during evolution; struc (—) = evolution in an unstructured environment; struc (+) = evolution in a structured environment. We used non-parametric Wilcoxon rank-sum test for comparisons relative to the ancestral wildtype, and Bayesian-based generalized linear mixed models to test for treatment effects (see Table S1). Solid black bars denote the median for each treatment.

Pyocyanin production, meanwhile, significantly dropped in all four environments (Fig. 2B), but more so in the presence than in the absence of the host (p_{host} = 0.038), while spatial structure had no effect ($p_{structure}$ = 0.981). The pattern of evolved protease production mirrored the one for pyocyanin (Fig. 2C): there was a significant overall decrease in protease production, with a significant host (p_{host} = 0.042), but no structure ($p_{structure}$ = 0.489) effect. Since neither pyocyanin nor proteases are necessary for growth in our media, consisting of a protein-digest, reduced expression could reflect selection against dispensable traits. During infections, however, these traits are known to be beneficial [30,31] and accelerated loss could thus be explained by cheating, as both secreted virulence factors could become exploitable inside the host. Finally, the clones' ability to attach to surfaces significantly increased in the presence of the host (p_{host} = 0.007) and in structured environments ($p_{structure}$ = 0.010), but decreased in the host-free unstructured environment (Fig. 2D). These findings indicate that attachment ability might be superfluous under shaken conditions, but could become important within the host to increase residence time.

While the phenotypic screens revealed altered virulence factor production levels, with significant host and environmental effects (Fig. 2), the virulence data suggest that there is no host effect, and spatial structure is the only determinant of virulence evolution (Fig 1). In the attempt to reconcile these apparently conflicting results, we first performed a principle component analysis (PCA) on population averages of the four virulence factor phenotypes (Fig. 3A). The PCA indicates that each treatment evolved in a different direction in the phenotype space, revealing that environmental and host factors indeed both seem to matter. This analysis further shows that the direction of phenotypic changes was aligned for some traits (pyocyanin and proteases), but opposed for others (pyocyanin/proteases vs. pyoverdine/attachment) (Fig. 3A, Fig. S2A-D).

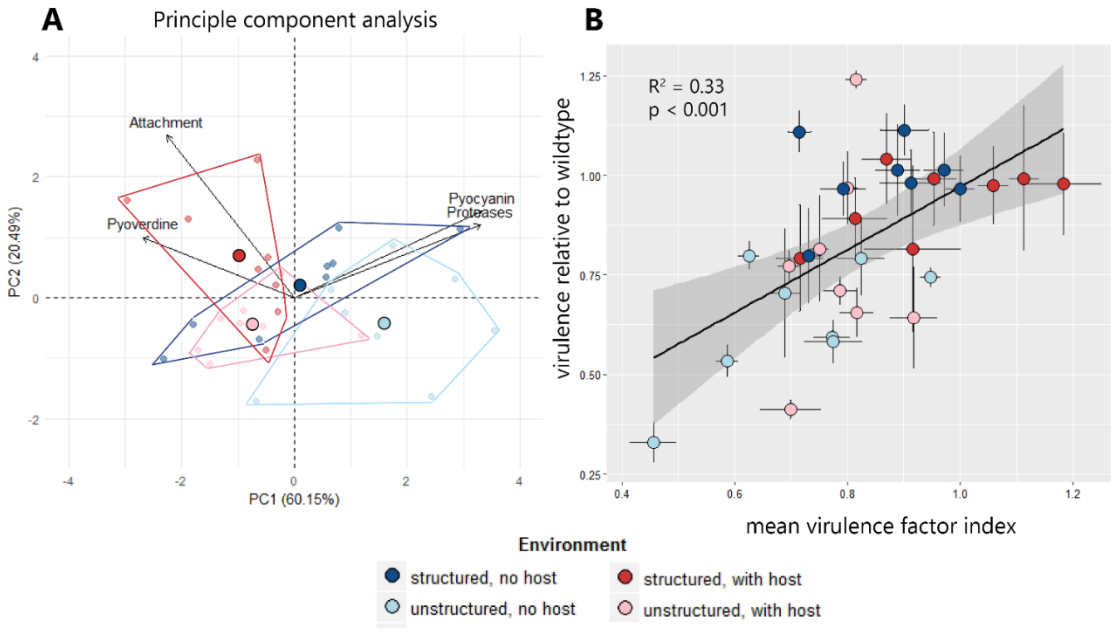

Fig. 3. The aggregate change in virulence factor production explains virulence evolution. (**A**) A principle component analysis (PCA) on the population-level changes in the production of four virulence factors (pyoverdine, pyocyanin, proteases, attachment) reveals divergent evolutionary patterns. For instance, analysis of the first two principal components (explaining 80.6 % of the total variation) shows complete segregation between populations evolved in unstructured host-free environments and structured environments with the host. Moreover, the PCA reveals that evolutionary change was aligned for some traits (aligned vectors for pyocyanin and proteases), but opposed for others (inversed vectors for pyoverdine versus pyocyanin/proteases). Small and large symbols depict individual population values and average values per environment, respectively. Polygons show the boundaries in phenotype space for each environment. (**B**) We found that the aggregate change in the production of all four virulence factors explained the evolution of virulence. To account for the aligned and opposing effects revealed by the PCA, we defined the "virulence factor index" as the average change in virulence factor production across all four traits, scaled relative to the ancestral wildtype. Symbols and error bars depict mean values per population and standard errors of the mean, respectively.

These opposing evolutionary directions could potentially cancel out and lead to a zero net effect on virulence. In line with this hypothesis, we found that phenotypic changes in a single virulence factor could not explain the evolved virulence (Fig. S3). In contrast, when taking the compensatory and aligned effects into account, the aggregate change in virulence phenotype explained a significant proportion of the variation observed in virulence evolution (Fig. 3B, $R^2 = 0.33$, $F(1,30) = 14.7$, $p < 0.001$; also see Fig. S4). Thus, it is the sum of virulence factor production change that explains the evolved virulence patterns, with both environmental and host factors playing a role.

Next, we examined whether the observed shifts in phenotypes can be linked to changes in the genotypes by sequencing the genome of 144 evolved clones, and calling SNPs and INDELs relative to the ancestral wildtype. We identified two mutational targets that explained many of the altered virulence factor phenotypes (Fig. 4, Fig. S5). Reduced pyoverdine production was significantly associated with mutations in the *pvdS* gene or its promoter region ($F(1,137) = 240.1$, $p < 0.0001$, Fig. 4A). PvdS (the iron-starvation sigma factor) controls pyoverdine synthesis, and mutations in this gene can lead to pyoverdine deficiency [32].

Moreover, there were significant links between reduced pyocyanin and protease production and mutations in *lasR*, encoding the regulator of the Las quorum-sensing (QS) system (pyocyanin: $F(1,137) = 18.76$, $p < 0.0001$; proteases: $F(1,137) = 16.04$, $p < 0.001$, Fig. 4B+C). In roughly half of the clones (pyocyanin: 51.3%, proteases: 45.6%), reduced production levels could be attributed to mutations in *lasR*. While the Las-system directly controls the expression of proteases, pyocyanin is only indirectly linked to this QS-system, via the two subordinate Rhl and PQS quorum sensing systems [33]. Finally, we found many mutations in genes involved in type IV pili synthesis (Fig. S5). Although type IV pili can be important for bacterial attachment to surfaces [34], there was no clear relationship between these mutations and the evolved attachment phenotypes (Fig.

S6). This is probably because surface attachment is a quantitative trait, involving many genes, and because we found both evolution of increased and decreased attachment abilities, which complicates the phenotype-genotype linking.

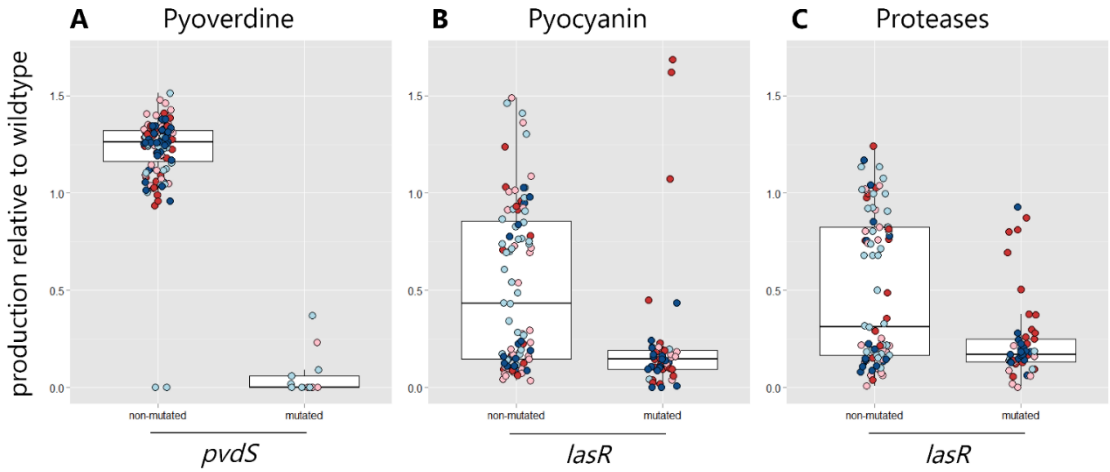

Fig. 4. Mutations in key regulatory genes underlie the loss of virulence factor production. The whole genomes of 144 evolved clones (36 per environment) were sequenced and SNPs and INDELs were called relative to the ancestral wildtype. Across clones, there was an accumulation of mutations in two regulatory genes (*pvdS* and *lasR*), which significantly correlated with the phenotypic changes observed for pyoverdine (**A**), pyocyanin (**B**) and protease (**C**) production (see Table S1). *pvdS* codes for the iron starvation sigma factor and all clones with mutations in this gene or its promoter showed significantly impaired pyoverdine production. LasR is the regulator of the Las-quorum-sensing system, which directly controls the expression of several proteases. All clones with *lasR* mutations showed reduced protease production. The LasR regulator has also downstream effects on the Rhl- and PQS quorum-sensing systems, which control pyocyanin production. Consistent with this view, virtually all clones with *lasR* mutations (93.8 %) showed decreased pyocyanin production. Although the genotype-phenotype match was nearly perfect for mutated clones, a considerable amount of clones also showed altered phenotypes without mutations in these two regulators, suggesting that some of the phenotypic changes are caused by mutations in yet unidentified genetic targets.

DISCUSSION

Our study demonstrates that virulence evolves rapidly in opportunistic pathogens and that similar virulence levels can arise based on completely different underlying phenotypic and genotypic changes. For instance, we found that evolution towards lower virulence can be entirely accidental in host-free unstructured media, driven by the spread of cheaters (pyoverdine non-producers) and the loss of non-essential traits (pyocyanin, proteases, surface attachment). In the presence of the host, virulence also decreased in unstructured media, but this time the decline was driven by the accelerated loss of pyocyanin and protease production, two virulence factors that are beneficial in the context of infections, whereas pyoverdine-cheating was no longer relevant. Finally, we demonstrate that different virulence factors can be under divergent selection, with their effects on virulence cancelling out. We observed this in our structured environments, where the reduction of pyocyanin and protease production was compensated by increased pyoverdine production and attachment capability. Altogether, our work highlights that virulence evolution is complex and multi-facetted, and that linking virulence evolution to the underlying mechanisms, in both the host and non-host environment, is key to predict evolutionary trajectories in opportunistic pathogens.

Our findings closely relate to previous work that has identified *lasR* as a key target of evolution in the context of chronic *P. aeruginosa* infections in the cystic fibrosis lung [16,35–38], in non-cystic fibrosis bronchiectasis [39], as well as in acute infections [24,40]. While the ubiquitous appearance of *lasR*-mutants was often interpreted as a specific host adaptation, we show here that *lasR*-mutants frequently arise even in the absence of a host, demonstrating that the associated virulence consequences are mostly accidental and not host specific. One explanation for the frequent occurrence of *lasR*-mutants is that *P. aeruginosa* is under selection to rewire its QS network, especially when

consistently growing at high densities, as occurring in infections and laboratory cultures [41].

Another important similarity to the work on clinical cystic fibrosis lung isolates is that we observed tremendous diversification during experimental evolution, and the co-existence of multiple different phenotypes and genotypes within each replicate. While this diversity might be transient in some cases, it highlights that an initially clonal infection can give rise to a diverse community, with multiple strains competing with each other within the host, as it was observed in CF lung communities [42,43].

Our study further reveals that most mutations affecting virulence phenotypes occur in the key regulators *pvdS* and *lasR*, and not in the genes encoding the virulence factors or enzymes required for their synthesis. Important to note, however, is that although almost all clones with mutations in these regulators had a phenotype (i.e. decreased pyoverdine, pyocyanin and protease production), the phenotype-to-genotype matching was not perfect (Fig. 4). For instance, we observed a high fraction of clones with reduced pyocyanin and protease production without a mutation in *lasR* (or any other known QS-related gene). This is maybe unsurprising given that the regulation of virulence factor expression is highly complex [19], such that other mutations, for instance those detected in hypothetical proteins (Fig. S5), could be responsible for the observed phenotypes. Along a similar line, while the loss of pyoverdine production was consistently associated with mutations in *pvdS*, the regulator of pyoverdine production, there was no association between the phenotypes showing increased pyoverdine production (Fig. 2A) and any type of mutation. We assume that the observed pyoverdine upregulation is a compensatory phenotypic response, as decreased pyocyanin and protease production are known to lower iron availability [44], which in turn might trigger increased pyoverdine production. Our insights on the complex phenotype-genotype mapping demonstrate

that care must be taken, when interpreting evolutionary change and virulence consequences based on genotypes alone.

In conclusion, our considerations show that there are no simple general predictions regarding virulence evolution across host-pathogen systems. Instead, there are the details of the abiotic environment and the biotic infectious context that together determine the direction of virulence evolution in both opportunistic bacterial (our study) and obligate viral pathogens [46]. For our specific case, we can attempt to link our findings to chronic *P. aeruginosa* cystic fibrosis infections, as we know that the CF lungs are typically filled with highly viscous mucus, providing a highly structured environment. Our results would suggest that cooperative virulence factor production is stabilized in this environment if untreated, while the breaking up the spatial structure through mucolysis could steer pathogen populations in CF lungs towards lower virulence.

MATERIALS AND METHODS

Strains and growth conditions. We used *Pseudomonas aeruginosa* wildtype strain PAO1 (ATCC 15692) constitutively expressing GFP (PAO1-*gfp*) for experimental evolution. The siderophore deficient mutant PAO1Δ*pvdD-gfp*, the quorum-sensing deficient mutants PAO1Δ*rhlR* and PAO1Δ*lasR* (S. Diggle, Georgia Institute of Technology, USA), and the biofilm deficient mutant MPAO1Δ*pelA*Δ*pslA* (M. Toyofuku, University of Zurich, Switzerland) were used as negative controls for phenotype screening. For overnight pre-culturing, we routinely used Luria Bertani (LB) medium and incubated the bacteria under shaking conditions (190 rpm) for 18-20 h, and optical density (OD) of bacterial cultures was determined in a Tecan Infinite M-200 plate reader (Tecan Group Ltd., Switzerland) at a wavelength of 600 nm, unless indicated otherwise. All experiments in this study were conducted at 25°C, except for the pre-culturing of the ancestral wildtype strain before the start of the experimental evolution

(see below). To generate iron-limited nutrient medium (RDM-Ch) suitable for bacterial and nematode co-culturing, we supplied low-phosphate NGM (nematode growth medium; 2.5 gL^{-1} BactoPeptone, 3 gL^{-1} NaCl, 5 mgL^{-1} Cholesterol, 25 mM MES buffer pH = 6.0, 1mM MgSO4, 1mM $CaCl_2$; adapted from [8] with the iron chelator 2,2'-Bipyridyl at a final concentration of 200 µM. For agar plates, liquid media was supplemented with 1.5% (m/V) agar. All chemicals were acquired from Sigma-Aldrich, Switzerland. *Caenorhabditis elegans* N2 wildtype nematodes were acquired from the Caenorhabditis Genetics Center (CGC). General nematode maintenance and generation of age-synchronized L4 nematodes was performed according to standard protocols [47].

Experimental evolution. Experimental evolution was conducted with a clonal population of PAO1-*gfp* bacteria as a starting point. For each of the four experimental treatments (agar plates with and without host, liquid culture with and without host), eight replicate lines were evolved independently. Throughout the experimental evolution, *C. elegans* was not allowed to co-evolve. Instead, fresh age-synchronized L4 stage nematodes were supplied at each transfer step. Since *P. aeruginosa* is highly virulent towards *C. elegans*, the vast majority of worms were dead before each transfer step. Each individual culture was visually checked for egg or L1 larvae development and we never observed any live larvae. We can therefore attest that the nematodes did not successfully reproduce during experimental evolution.

At the start of the experimental evolution, overnight cultures of PAO1-*gfp* were grown under shaken conditions (190-200 rpm) at 37°C for 18 h, washed with NaCl (0.85%) and adjusted to an OD600 of 1.0. After this point, all steps throughout the experimental evolution were conducted at 25°C. For evolution on agar plates and for each replicate line, 50 µL of cell suspension were spread onto a small RDM-Ch agar plate (diameter 60 mm). Approximately 100 age-synchronized L4 stage *C. elegans* nematodes were then added to each plate in the treatment "agar plate

with host", and all plates were incubated for 48 h before the first transfer. For evolution in liquid cultures, the same OD-adjusted bacterial suspensions were diluted 10^{-4} into 5 mL of liquid RDM-Ch in 15 mL culture tubes. Approximately 2500 age-synchronized L4 stage *C. elegans* nematodes were then added to each tube for the treatment "liquid culture with host", and all tubes were incubated for 48 h under "rolling" conditions (160 rpm) in a horizontal position to avoid clumping of the worms.

Transfers of bacteria to fresh nutrient medium and, if applicable, addition of fresh nematodes to the samples were conducted every 48 h and executed as follows. For all agar plates, bacteria were replica-plated to a fresh RDM-Ch plate, using a custom made replica tool covered in sterilized velvet. In the treatment "agar plate with host", the plates containing the nematodes from the previous round were then rinsed off the plate with sterile NaCl (0.85%), washed thoroughly to avoid additional transfer of bacteria, and 10% of the nematode suspension was transferred to the new plate. Since *P. aeruginosa* is highly virulent towards *C. elegans*, the transferred worms were carcasses. A fresh batch of ~100 synchronized L4 stage nematodes was then added to the plates. For the "liquid culture without host" treatment, 50 µL of the culture was used to inoculate 4.95 mL of fresh RDM-Ch medium. For the "liquid culture with host" treatment, culture tubes were centrifuged slowly (~200 g, 5 min) to pellet the nematodes, and 50 µL of the supernatant (still containing the bacteria) was used to inoculate 4.95 mL of fresh RDM-Ch medium. The pelleted nematodes were then washed thoroughly with sterile NaCl (0.85%), and 10% of the nematode suspension was transferred to the new culture tube. Analogous to the agar treatment, most transferred worms were carcasses due to the high virulence levels of *P. aeruginosa*. A fresh batch of ~2500 synchronized L4 nematodes was then added to the tubes.

The number of viable bacteria transferred through replica-plating corresponded approximately to a 1:100 dilution, and was therefore

equivalent to the dilution achieved in the liquid cultures. In total, 30 transfers were conducted, corresponding to approximately 200 generations of bacterial evolution. At the end of the experimental evolution, evolved populations were frozen for further analysis as follows. For the two agar plates treatments, the bacterial lawn was washed off with sterile NaCl (0.85%), mixed vigorously, diluted 10^{-3} into 3 mL of liquid LB medium in 6-well plates, and incubated under shaken conditions (100 rpm) for 18 h. For the "liquid culture with host" treatment, culture tubes were first centrifuged slowly (~200 g, 5 min) to pellet the nematodes. Then, 25 µL of the supernatant (containing bacteria) was used to inoculate 2.475 mL liquid LB medium in 6-well plates. For the "liquid culture without host" treatment, 25 µL of the bacterial culture was used to inoculate 2.475 mL liquid LB medium in 6-well plates. All plates were then incubated under shaken conditions (100 rpm) for 18 h. Finally, 900 µL of each well was mixed 1:1 with sterile glycerol (85%) and frozen at -80°C in separate cryotubes.

Killing assays for virulence measurements. Population level virulence was assessed in two different killing assays, namely in liquid culture and on agar plates, representing the two different environments the different bacterial populations evolved in. Populations were separately tested both in the environment they evolved in (populations evolved on agar plates tested on agar plates, and populations evolved in liquid culture tested in liquid culture), and in the respective reciprocal environment (populations evolved in liquid culture tested on agar plates, and vice versa). All killing assays were conducted at 25°C.

For killing assays in liquid culture, evolved bacterial populations and the ancestral wildtype strain were re-grown from freezer stocks in LB medium overnight, washed with sterile NaCl (0.85%), adjusted to OD600=1.0 and diluted 10^{-4} into 5 mL of liquid RDM-Ch medium in a 15 mL culture tube. Three replicate tubes were inoculated per tested population. After an incubation period of 48 h (shaken conditions, 160-165 rpm), the OD600

was measured and cells were pelleted through centrifugation. A volume ≤ 500 µL of the supernatant was removed, corresponding to the volume containing ~2500 synchronized L4 nematodes that were subsequently added. Culture tubes were then incubated for 48 h under "rolling" conditions at 160 rpm in a horizontal position to avoid clumping of the worms. At 24 h and 48 h after adding the nematodes, the level of virulence was determined by counting the fraction of dead worms. Small aliquots were taken from the main culture and dropped onto an NGM plate. After a short drying period, nematodes were prodded repeatedly with a metal rod and counted as dead if they did not show any signs of movement. Dead worms were immediately removed to avoid double counting.

For killing assays on agar plates, evolved bacterial populations and the ancestral wildtype strain were re-grown from freezer stock in LB medium overnight, washed with sterile NaCl (0.85%), adjusted to OD600=1.0 and 50 µL were spread on RDM-Ch agar plates. Six replicate plates were inoculated per tested population. Plates were incubated for 48 h, and an aliquot of synchronized L4 nematodes suspended in liquid was then added to the plates. The nematodes had been previously starved on empty NGM plates for 24 h. The starting number of nematodes ranged from 20 to 60 worms per plate and was immediately determined by manual counting. Plates were then incubated further and at 24 h and 48 h after adding the nematodes, the level of virulence was determined by counting the number of dead worms on the plates, as described for the killing assay in liquid culture.

For both killing assays, each individual liquid culture and plate was visually checked for egg or L1 larvae development and we never observed any live larvae. We can therefore attest that the nematodes did not successfully reproduce during experimental evolution.

Isolation of single clones. To isolate single clones, evolved bacterial populations were re-grown from freezer stock in 3 mL LB medium for 20 h (160 rpm) and adjusted to OD600=1.0. Then, 200 µL of 10^{-6} and 10^{-7} dilutions were spread on large LB agar plates (diameter 150 mm), and plates were incubated at room temperature (~20-25°C) for 48 h. Twenty colonies were then randomly picked for each population and inoculated into 100 µL LB medium in a 96-well plate. Plates were incubated for 24 h under shaken conditions (165 rpm) before adding 100 µL sterile glycerol (85%) to each well, sealing the plates with adhesive foil and freezing at -80°C. A total number of 640 clones was isolated this way, and each was subjected to four different phenotypic screens for virulence factor production.

Phenotypic screen for virulence factor production

Pyoverdine production. Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, cultures were first diluted 10^{-2} in NaCl (0.85%) and then 10^{-2} into liquid RDM-Ch to a final volume of 200 µL in a 96-well plate. Plates were then incubated for 24 h under shaken conditions (165 rpm) and OD600 and pyoverdine-specific fluorescence (emission 400 nm, excitation 460 nm) were measured in a plate reader through single endpoint measurements. Multiple wells inoculated with the ancestral wildtype as well as blank medium controls were included in every plate. Additionally, the pyoverdine knockout mutant PAO1-Δ*pvdD-gfp* was included as a negative control for pyoverdine fluorescence.

Pyocyanin production. Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, cultures were first diluted 10^{-2} in NaCl (0.85%) and then 10^{-2} into liquid LB to a final volume of 1 mL in 24-well plates. Plates were then incubated for

24 h under shaken conditions (165 rpm). The well content was then transferred to 1.5 mL reaction tubes, vortexed thoroughly, and centrifuged to pellet bacterial cells. From each tube, three aliquots of 150 µL of the cell-free supernatant were then transferred to 96-well plates, and pyocyanin was quantified by measuring OD at 691 nm in a plate reader. Multiple wells inoculated with the ancestral wildtype as well as blank medium controls were included in every plate. Additionally, the Rhl-quorum-sensing deficient knockout mutant PAO1-Δ*rhlR* was included as a negative control for pyocyanin production.

Protease production. Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, 1 µL of bacterial culture was dropped into a single well of a 24-well plate filled with skim milk agar (5 gL^{-1} LB, 4% (m/V) skim milk powder, 15 gL^{-1} agar) and plates were incubated for 20 h. Pictures of the plates were then taken with a standard digital camera and analyzed with the Image Analysis Software *ImageJ* [48]. The diameter of the clear halo around the bacterial colony and the diameter of the colony itself was measured, and protease production was calculated using the following formula:

$$relative\ protease\ production = \frac{(diameter(halo) - diameter(colony))}{diameter(colony)}.$$

Multiple wells inoculated with the ancestral wildtype as well as blank medium controls were included in every plate. Additionally, the Las-quorum-sensing deficient knockout mutant PAO1-Δ*lasR* was included as a negative control for protease production.

Surface attachment. Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, the air liquid biofilm was manually removed from the surface with a sterile pipette tip. Cultures were then diluted 10^{-2} into 100 µL LB medium in a 96-

well round bottom plate (No. 83.3925.500, Sarstedt, Germany) and incubated under static conditions for 24 h. After removal of the air liquid biofilm, the growth medium containing the planktonic cells was transferred to a fresh flat-bottom 96-well plate and OD was measured at 550 nm in a plate reader. In the plate containing the cells attached to the plastic surface, 100 µL of crystal violet (0.1%) was added to each well and plates were incubated at room temperature for 30 min. Then, the wells were carefully washed several times with ddH_2O, left to dry at room temperature for 30 min, and 120 µL DMSO was added to each well before a final incubation step of 20 min at room temperature. Finally, OD was measured at 570 nm in a plate reader, and surface attachment was quantified by calculating the "Biofilm Index" (OD570/OD550) for each well [49]. Multiple wells inoculated with the ancestral wildtype as well as blank medium controls were included in every plate. Additionally, the knockout mutant MPAO1-Δ*pelA*-Δ*pslA* was included as a negative control for surface attachment.

Calculation of the "virulence factor index". We defined a virulence factor index $v = \Sigma\, r_i \,/\, n$, where r_i-values represent the average virulence factor production scaled relative to the ancestral wildtype for the *i*-th virulence factor (*i* = pyoverdine, pyocyanin, proteases, surface attachment), and *n* is the total number of virulence factors. A clone with wildtype production levels for all four virulence factors measured would have a virulence index of ~1, whereas a clone with mostly lowered or absent production would have a virulence index closer to 0. For statistical analyses and the generation of Fig. 3B and Fig. S3, we used the average virulence index across clones for each population.

Whole genome sequencing and SNP calling

Selection of clones to sequence. To select populations from which to select clones for sequencing, we first chose all populations that showed a decrease in virulence, and then added randomly chosen populations to cover all four treatments in a balanced way (four sequenced populations for each treatment), leading to a total of 16 selected evolved populations. From these, we selected 9 clones per population according to the following scheme: first, we tried to get at least one clone that showed no phenotypic differences to the ancestral wildtype with regards to pyoverdine and pyocyanin production. Then, we tried to get clones with a marked decrease in pyoverdine and/or pyocyanin production. Finally, we filled up the list with randomly chosen clones.

Genomic DNA isolation. Clones were re-grown from freezer stocks in 3 mL LB medium in 15 mL culture tubes at 190 rpm for 20-24 h. Genomic DNA was then extracted from 1 mL of culture using the *GenElute™ Bacterial Genomic DNA Kit* (Sigma-Aldrich, Switzerland) according to the manufacturer's instructions. At the final step of the isolation protocol, the DNA was eluted in TRIS-HCl without the addition of EDTA to avoid interference with sequencing library preparation. DNA concentration was quantified using the *QuantiFluor® dsDNA System* (Promega, Switzerland) according to the manufacturer's instructions, and diluted to a concentration of 10 ng/µL for use in subsequent library preparation.

Preparation of sequencing library and whole genome sequencing. Sequencing libraries were constructed using the Nextera XT Kit (Illumina, USA). Briefly, 0.8 ng of gDNA per sample was tagmented at 55 °C for 10 min. Libraries were dual-indexed and amplified in the subsequent library PCR. Sequencing libraries were cleaned up using cleanNA SPRI beads (GC biotech, Netherlands) according to the manufacturer's protocol.

Next, DNA concentration was quantified using the *QuantiFluor® dsDNA System* (Promega, Germany) and equal amounts of library per sample pooled. Finally, the molarity of the library pool was determined using the *dsDNA High Sensitivity Assay* for the *Bioanalyzer 2100* (Agilent Technologies, Germany). Sequencing was performed 2x150 bp by Microsynth (Balgach, Switzerland) on a NextSeq500 (Illumina, USA).

SNP calling. Demultiplexed reads were aligned to the *P. aeruginosa* PAO1 reference genome using bowtie2 in local-sensitive mode [50]. PCR duplicates were removed using "picard" tools (broadinstitute.github.io/picard). Variants were called using "samtools" (v0.1.19), "mpileup" and "bcftools" [51]. Variants were filtered with default parameters using "samtools" and "vcfutils". Variant effects were predicted using SnpEff (version 4.1d) [52]. Annotated variant calls were only retained if more than 80% of reads contained the alternate base. All variants already occurring in the ancestral wildtype strain were discarded for analysis of the evolved clones. Of the 144 sequenced clones, three had to be discarded before analysis due to low coverage, and one for likely being a mixture of two different genotypes due to contamination.

Statistical Analysis. We used linear models and linear mixed models for statistical analyses using R 3.2.2 [53]. In cases where data distributions did not meet the assumptions of linear models, we performed non-parametric Wilcoxon rank sum tests. To test whether evolved virulence factor production in single clones depended on the environment they evolved in, we used Markov-chain Monte Carlo generalized linear mixed models (MCMCglmm) in a Bayesian framework [54]. In this context, *p* represents the posterior probability associated with a fixed effect, and as such is not a "classical" frequentist p-value, but provides the same kind of information. For all results analyzed with MCMCglmm, we ran the analyses

at least five consecutive times to confirm that p-values were consistently < 0.05. Principal component analysis (PCA) was conducted using the 'FactoMineR' [55] and 'factoextra' packages (https://CRAN.R-project.org/package=factoextra). Detailed information on the results of all statistical tests associated with this publication can be found in Supplementary Table S1.

ACKNOWLEDGEMENTS

We thank Swati Parekh for assistance in genomic analysis, Isa Moreno for help with the nematode killing assays, Ruben Dezeure for statistical advice, and Stuart West for comments on the manuscript.

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6.2 Supporting Material

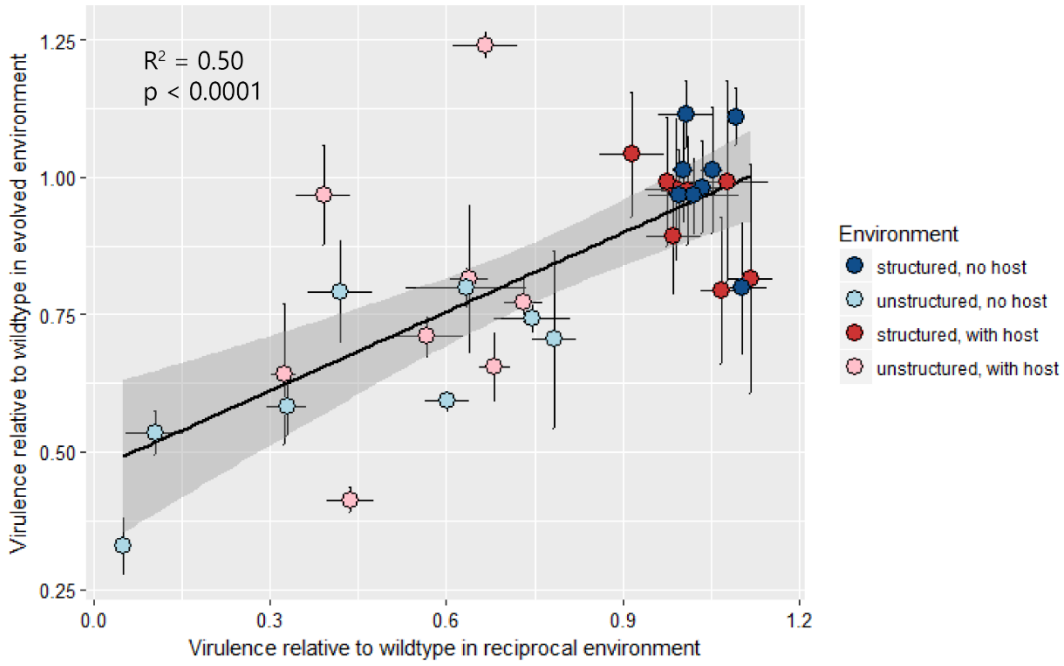

Fig. S1. Virulence measured in two different assays yields highly similar results. 32 evolved populations were tested for their virulence towards the nematode *C. elegans*. *Y* axis shows virulence tested in the environment the populations evolved in: populations that evolved on agar plates tested on agar plates, populations that evolved in liquid culture tested in liquid culture. *X* axis shows virulence when tested in the reciprocal environment: populations that evolved on agar plates tested in liquid culture, populations that evolved in liquid tested on agar plates. Virulence was quantified as percent nematodes killed at 24 h post infection, scaled to the ancestral wildtype, and averaged across three to six replicates per population. Individual dots represent average virulence of a population. Error bars denote the standard error of the mean. See Table S1 for details on statistical analysis.

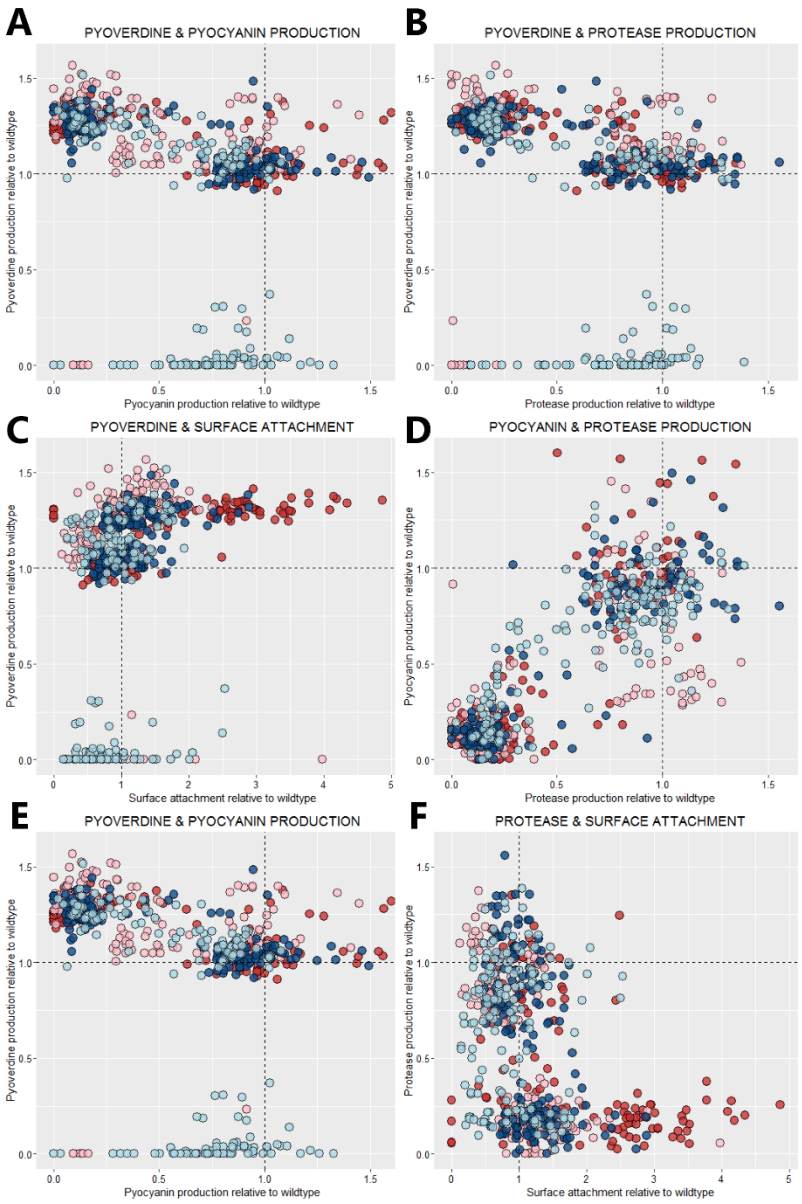

Fig. S2. Pairwise comparisons of the production of different secreted virulence factors. 640 evolved clones were tested for their expression of four secreted virulence factors. Plots each show pairwise comparisons of the production of two virulence factors for all clones. All values scaled to the ancestral wildtype. Colours represent the different environments the populations evolved in. Pyoverdine production against pyocyanin production (**A**), protease production (**B**) and surface attachment (**C**); pyocyanin against protease production (**D**) and surface attachment (**E**); protease production against surface attachment (**F**).

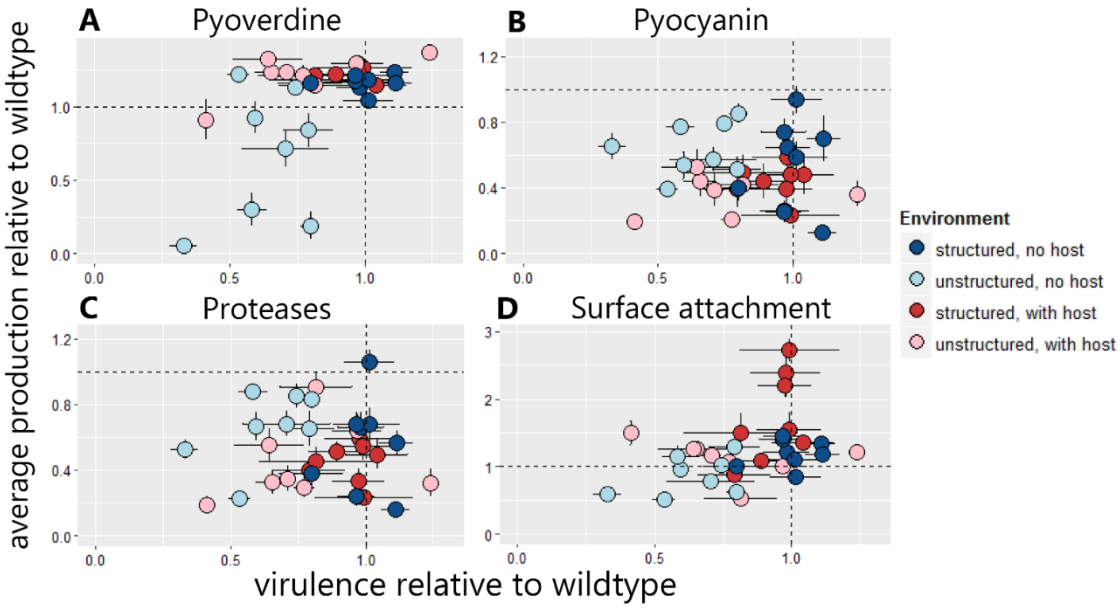

Fig. S3. No single virulence factor can fully explain evolved virulence levels. Virulence levels of 32 evolved populations were determined and plotted against average virulence factor production in 20 evolved clones per population. All values scaled to the ancestral wildtype. Error bars represent the standard error of the mean. (**A**) Virulence plotted against pyoverdine production. (**B**) Virulence plotted against pyocyanin production. (**C**) Virulence plotted against protease production. (**D**) Virulence plotted against surface attachment. Colours represent the different environments the populations evolved in.

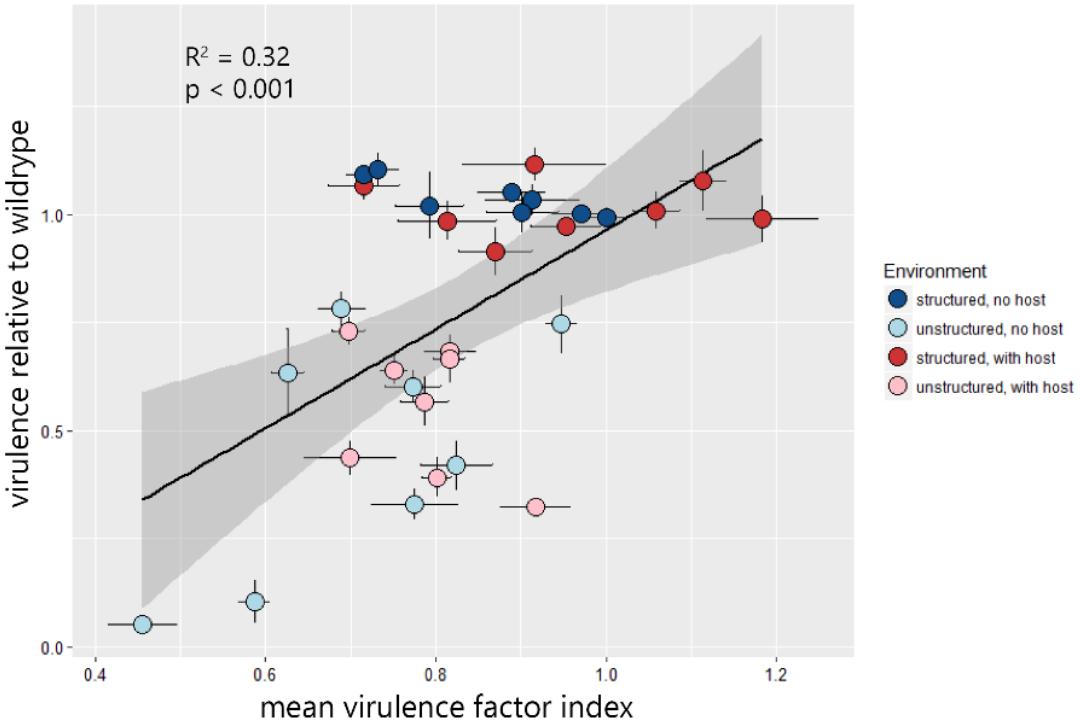

Fig. S4. Decrease in virulence explained by combining the expression profile of four virulence factors in evolved clones. For each of 640 evolved clones, the "virulence factor index" was calculated by summing over production levels of four secreted virulence factors and scaling to the ancestral wildtype. *X* axis shows average virulence indices, while *y* axis shows average virulence levels scaled to the wildtype. Individual dots represent average values across 20 clones for each evolved population, coloured by the environment they evolved in. Virulence was tested in the reciprocal environment: populations that evolved on agar plates tested in liquid culture, populations that evolved in liquid tested on agar plates. Virulence was quantified as percent nematodes killed at 24 h post infection, scaled to the ancestral wildtype, and averaged across three to six replicates per population. Error bars denote the standard error of the mean. See Table S1 for details on statistical analysis.

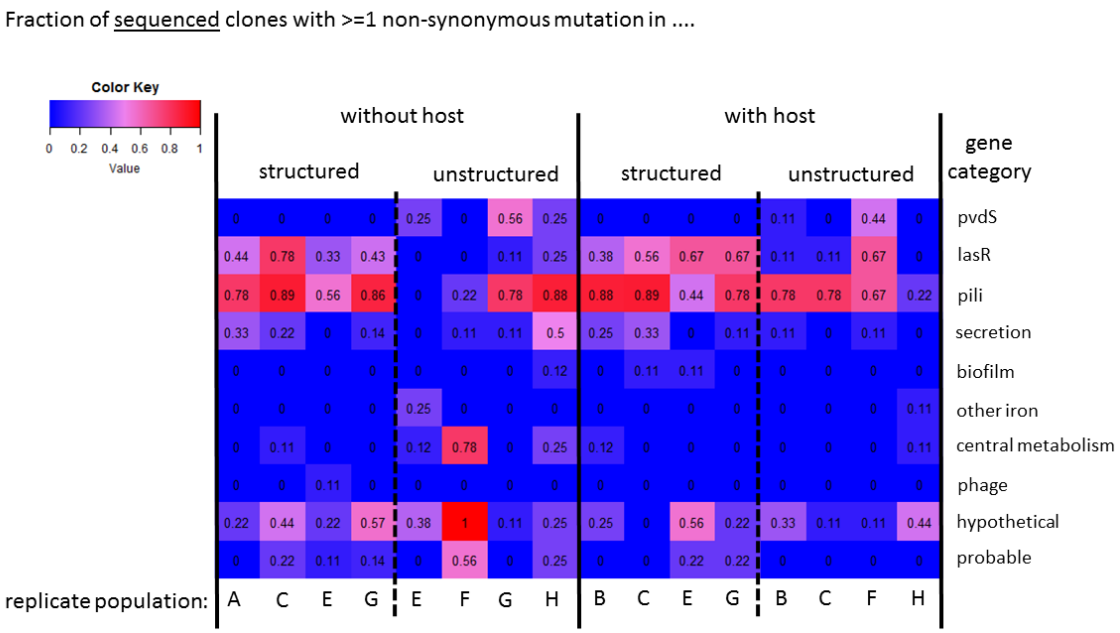

Fig. S5. Heatmap showing mutations in different functional categories and single genes. Whole genome sequencing was performed on 144 evolved clones and SNPs and INDELs were identified in genes belonging to different functional categories. Heatmap colouring represents fraction of clones with ≥ 1 non-synonymous mutation in respective gene or gene group, per replicate evolved population. Seven to nine clones were sequenced per population.

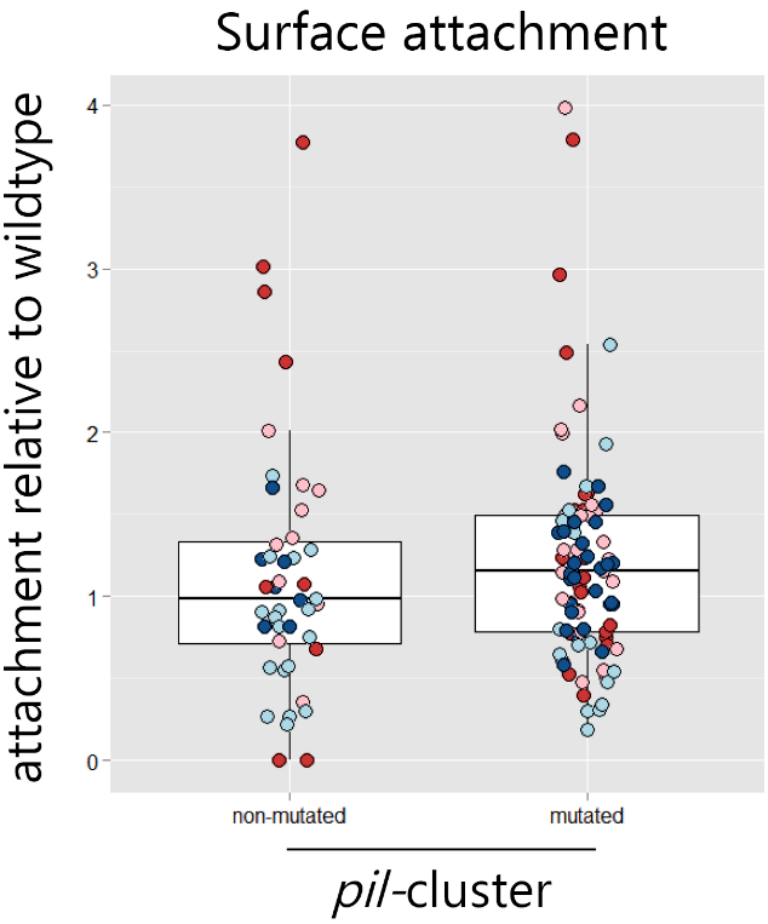

Fig. S6. Surface attachment not affected by mutations in the *pil* gene cluster. Whole genome sequencing was performed on 144 evolved clones, and SNPs and INDELs in genes related to surface attachment were tested for their effect on surface attachment levels. Clones with ≥1 detected SNP or INDEL in the respective gene are labelled "mutated", clones with no SNPs or INDELs detected in this region are labelled "non-mutated". Y axis values are scaled to surface attachment levels of the ancestral wildtype. We did not detect a different in surface attachment levels in clones with mutations in the *pil* gene cluster (*pilM, pilQ, pilO, pilU, pilD, pilA, pilZ, pilY1, pilW, pilN, pile, pilB, pilS, pilR, pilT, pilG*) when compared to clones without mutations in these genes (p = 0.64). See Table S1 for details on statistical analysis.

Table S1A. Supplementary information on statistical analyses conducted in this study.

Virulence (Fig.1B+C)										
comparing treatment factors										
	statistical test	**formula/syntax**	**degrees of freedom**	**P value**						
virulence measured in "evolved" environments	linear mixed model	virulence ~ host(yes/no) * structure(high/low) + (1 | replicate population)	host 18.58 |struc 24.65 |host:structure 24.81	host 0.3985 |struc 0.0454 |host:structure 0.0670						
virulence measured in "reciprocal" environments	linear mixed model	virulence ~ host(yes/no) * structure(high/low) + (1 | replicate population)	host 45.59 |struc 35.82 |host:structure 35.82	host 0.831 |struc 1.77e-05 |host:structure 0.381						
treatments - comparison to ancestor										
	statistical test	**formula/syntax**	**W(with host, high structure)**	**p-value(with host, high structure)**	**W(with host, low structure)**	**p-value(with host, low structure)**	**W(no host, high structure)**	**p-value(no host, high structure)**	**W(no host, low structure)**	**p-value(no host, low structure)**
virulence measured in "evolved" environments	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved virulence, virulence of ancestor)	598	0.423	181	0.005637	631	0.533	62	8.828E-07
virulence measured in "reciprocal" environments	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved virulence, virulence of ancestor)	390	0.6075	99.5	5.706E-10	444.5	0.287	94	5.164E-10
Virulence factors (Fig.2A-D)										
comparing treatment factors										
	statistical test	**formula/syntax**	**p-value**							
pyoverdine	Markov chain Monte Carlo generalized linear mixed model	pyoverdine production ~ structure(high/low) + host(yes/no)	host:struc 0.0272*							
pyocyanin	Markov chain Monte Carlo generalized linear mixed model	pyocyanin production ~ structure(high/low) + host(yes/no)	host 0.0376* |struc 0.9808							
proteases	Markov chain Monte Carlo generalized linear mixed model	protease production ~ structure(high/low) + host(yes/no)	host 0.0422* |struc 0.4889							
surface attachment	Markov chain Monte Carlo generalized linear mixed model	surface attachment ~ structure(high/low) + host(yes/no)	host 0.0104* |struc 0.0072*							
			*repeated analysis consistently yielded p < 0.05							
treatments - comparison to ancestor										
	statistical test	**formula/syntax**	**W(with host, high structure)**	**p-value(with host, high structure)**	**W(with host, low structure)**	**p-value(with host, low structure)**	**W(no host, high structure)**	**p-value(no host, high structure)**	**W(no host, low structure)**	**p-value(no host, low structure)**
pyoverdine	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved pyoverdine production, pyoverdine production in ancestor)	11381	8.80E-16	12034	8.80E-16	11180	8.80E-16	6587	0.713
pyocyanin	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved pyocyanin production, pyocyanin production in ancestor)	1759.5	4.48E-14	894	8.80E-16	1857.5	1.25E-13	1362.5	8.80E-16
proteases	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved protease production, protease production in ancestor)	1276	8.80E-16	1678	1.21E-14	2062	6.00E-12	2353.5	2.75E-10
surface attachment	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved attachment, attachment in ancestor)	3433	0.002362	3071	0.07524	3145	0.04166	1833	0.01135

Table S1B. Supplementary information on statistical analyses conducted in this study.

Virulence (Fig.1B+C)										
comparing treatment factors										
	statistical test	**formula/syntax**	**degrees of freedom**	**P value**						
virulence measured in "evolved" environments	linear mixed model	virulence ~ host(yes/no) * structure(high/low) + (1 | replicate population)	host 18.58 | struc 24.65 | host:structure 24.81	host 0.3985 | struc 0.0454 | host:structure 0.0670						
virulence measured in "reciprocal" environments	linear mixed model	virulence ~ host(yes/no) * structure(high/low) + (1 | replicate population)	host 45.59 | struc 35.82 | host:structure 35.82	host 0.831 | struc 1.77e-05 | host:structure 0.381						
treatments - comparison to ancestor										
	statistical test	**formula/syntax**	**W(with host, high structure)**	**p-value(with host, high structure)**	**W(with host, low structure)**	**p-value(with host, low structure)**	**W(no host, high structure)**	**p-value(no host, high structure)**	**W(no host, low structure)**	**p-value(no host, low structure)**
virulence measured in "evolved" environments	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved virulence, virulence of ancestor)	598	0.423	181	0.005637	631	0.533	62	8.828E-07
virulence measured in "reciprocal" environments	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved virulence, virulence of ancestor)	390	0.6075	99.5	5.706E-10	444.5	0.287	94	5.164E-10
Virulence factors (Fig.2A-D)										
comparing treatment factors										
	statistical test	**formula/syntax**	**p-value**							
pyoverdine	Markov chain Monte Carlo generalized linear mixed model	pyoverdine production ~ structure(high/low) + host(yes/no)	host:struc 0.0272*							
pyocyanin	Markov chain Monte Carlo generalized linear mixed model	pyocyanin production ~ structure(high/low) + host(yes/no)	host 0.0376* | struc 0.9808							
proteases	Markov chain Monte Carlo generalized linear mixed model	protease production ~ structure(high/low) + host(yes/no)	host 0.0422* | struc 0.4889							
surface attachment	Markov chain Monte Carlo generalized linear mixed model	surface attachment ~ structure(high/low) + host(yes/no)	host 0.0104* | struc 0.0072*							
			*repeated analysis consistently yielded p < 0.05							
treatments - comparison to ancestor										
	statistical test	**formula/syntax**	**W(with host, high structure)**	**p-value(with host, high structure)**	**W(with host, low structure)**	**p-value(with host, low structure)**	**W(no host, high structure)**	**p-value(no host, high structure)**	**W(no host, low structure)**	**p-value(no host, low structure)**
pyoverdine	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved pyoverdine production, pyoverdine production in ancestor)	11381	8.80E-16	12034	8.80E-16	11180	8.80E-16	6587	0.713
pyocyanin	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved pyocyanin production, pyocyanin production in ancestor)	1759.5	4.48E-14	894	8.80E-16	1857.5	1.25E-13	1362.5	8.80E-16
proteases	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved protease production, protease production in ancestor)	1276	8.80E-16	1678	1.21E-14	2062	6.00E-12	2353.5	2.75E-10
surface attachment	Wilcoxon rank sum test (Mann-Whitney U test)	wilcox.test(evolved attachment, attachment in ancestor)	3433	0.002362	3071	0.07524	3145	0.04166	1833	0.01135

7 PROJECT 3 – Evolution of cooperation

"No snowflake in an avalanche ever feels responsible."

Stanislaw Jerzy Lec, Polish poet (1909-1966)

7.1 The path to re-evolve cooperation is constrained in *Pseudomonas aeruginosa*

This research has been submitted to a peer reviewed journal.

A preprint is available at https://doi.org/10.1101/163048

The path to re-evolve cooperation is constrained in *Pseudomonas aeruginosa*

Elisa T. Granato[1], Rolf Kümmerli[1]

[1]Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland.

ABSTRACT

Background. A common form of cooperation in bacteria is based on the secretion of beneficial metabolites, shareable as public good among cells at the group level. Because cooperation can be exploited by "cheat" mutants, which contribute less or nothing to the public good, there has been great interest in understanding the conditions required for cooperation to remain evolutionarily stable. In contrast, much less is known about whether cheats, once fixed in the population, are able to revert back to cooperation when conditions change. Here, we tackle this question by subjecting experimentally evolved cheats of *Pseudomonas aeruginosa*, partly deficient for the production of the iron-scavenging public good pyoverdine, to conditions previously shown to favor cooperation.

Results. Following approximately 200 generations of experimental evolution, we screened 720 evolved clones for changes in their pyoverdine production levels. We found no evidence for the re-evolution of full cooperation, even in environments with increased spatial structure, and reduced costs of cooperation – two conditions that have previously been shown to maintain cooperation. In contrast, we observed selection for complete abolishment of pyoverdine production. The patterns of complete trait degradation were likely driven by "cheating on cheats" in unstructured, iron-limited environments where pyoverdine is important for growth, and selection against a maladaptive trait in iron-rich environments where pyoverdine is superfluous.

Conclusions. Our study shows that the path to re-evolve cooperation seems constrained. One reason might be that the number of mutational targets potentially leading to reversion is limited. Alternatively, it could be that the selective conditions required for revertants to spread from rare are much more stringent than those needed to maintain cooperation.

INTRODUCTION

Bacterial life predominantly takes place in diverse communities, where individual cells are constantly surrounded by neighbors. While high cell density and diversity can create strong competition in the struggle for nutrients and space [1,2], it can also promote stable networks of cooperation [3,4]. A common way for bacteria to cooperate is through the secretion of nutrient-scavenging metabolites, which are shared as "public goods" in the community. Public goods cooperation is thought to increase nutrient uptake rate, and results in the costs and benefits of public goods being shared among producer cells. Although beneficial for the collective as a whole, public goods cooperation can select for "social cheats": mutants that lower or abolish their investment into public good production, but still reap the benefits of nutrient uptake [5,6].

The undermining of public goods cooperation by cheats has spurred an entire field of research, examining the conditions required for cooperation to be maintained in the population. In contrast, the question of how public goods cooperation evolves in the first place has received much less attention. The main question is: will the conditions that have been shown to maintain cooperation also promote the evolution of cooperation? Here, we tackle this question by examining whether bacteria that have evolved low levels of cooperation in a previous experiment can evolve back to normal levels of cooperation under conditions that are known to be favorable for cooperation. We use pyoverdine, an iron-scavenging siderophore secreted by the opportunistic pathogen *Pseudomonas aeruginosa*, as our model cooperative trait. Pyoverdine is the main siderophore of *P. aeruginosa*, and is secreted into the environment in response to iron limitation. Pyoverdine acts as a shareable public good that can be exploited by non-producing cheats that possess the matching receptor for uptake [7,8].

We consider three factors that could determine whether cooperation can re-evolve or not. The first factor is the spatial structure of the environment. Previous work revealed that increased spatial structure maintains cooperation because it reduces pyoverdine diffusion and cell dispersal. In other words, spatial structure ensures that pyoverdine sharing occurs predominantly among producer cells [9,10]. The second factor involves the relative costs and benefits of pyoverdine production [8,11]. In the absence of significant spatial structure, it was shown that cheats enjoyed highest relative fitness advantages under severe iron limitation when pyoverdine is expressed at high levels (i.e. high costs). Conversely, cooperation was maintained at intermediate iron limitation when pyoverdine is still important for growth, yet its investment is reduced (i.e. lower costs). Finally, we examine whether the genetic background of cheats is an important determinant of whether cooperation can re-evolve. Previous studies [7,12; Granato ET, Ziegenhain C & Kümmerli R, unpublished] observed the evolution of two types of cheats with greatly decreased pyoverdine production. The first type of cheat has a point mutation in *pvdS*, the gene encoding the sigma factor regulating pyoverdine production [13], whereas the second type of cheat has a point mutation in the promoter region of *pvdS*. While the two types of mutations might differ in their likelihood to revert back to cooperation, both could principally do so, because their pyoverdine biosynthesis cluster is intact [14], and a single point mutation in regulatory elements could lead to reversion.

We conducted experimental evolution in replicated populations with the two types of pyoverdine deficient strains across three levels of iron limitations and two habitats, differing in their level of spatial structuring. Based on social evolution theory, we predict the reversion to full cooperation whenever Hamilton's rule [15] – $rB > C$ – is satisfied. While r is the relatedness between the actor and the recipient, C is the cost to the actor performing cooperation, and B is the benefit gained by the

individual receiving cooperation. In our treatments, we vary *r* by manipulating the degree of spatial structure and *C/B* by manipulating the level of iron limitation. Accordingly, we predict that increased spatial structure and/or moderate investments into pyoverdine production should be most conducive for the re-evolution of cooperation. Moreover, we also envisage the possibility of pyoverdine production to degrade even further. This seems plausible because the mutated clones still produce some pyoverdine, and thus, there is room for further exploitation by *de novo* mutants that make even less. We predict this to happen under low spatial structure, and high pyoverdine investment levels. Finally, pyoverdine could also be degraded due to disuse [16], especially under conditions of high iron availability where pyoverdine is not required.

RESULTS

Characterization of the ancestral pyoverdine deficient strains

We first characterized the strains *pvdS*_gene and *pvdS*_prom for their pyoverdine production and growth dynamics (Fig. 1) before they were subjected to experimental evolution (Fig. 2). These two mutants themselves spontaneously arose and spread during a previous experimental evolution study (Granato ET, Ziegenhain C & Kümmerli R, unpublished). Their entire genomes had been re-sequenced and analyzed. Those analyses revealed that both *pvdS*_gene and *pvdS*_prom carried non-synonymous mutations that are directly associated with their reduced pyoverdine investment levels (Fig. 1a). Strain *pvdS*_gene has a point mutation (G>C) in the *pvdS* gene that leads to an amino acid change (Met135Ile), and thus to a modified iron-starvation sigma factor PvdS. A modified PvdS presumably has lower affinity to the RNA-polymerase, a complex that directly controls the expression of the non-ribosomal peptide synthesis machinery required to build pyoverdine.

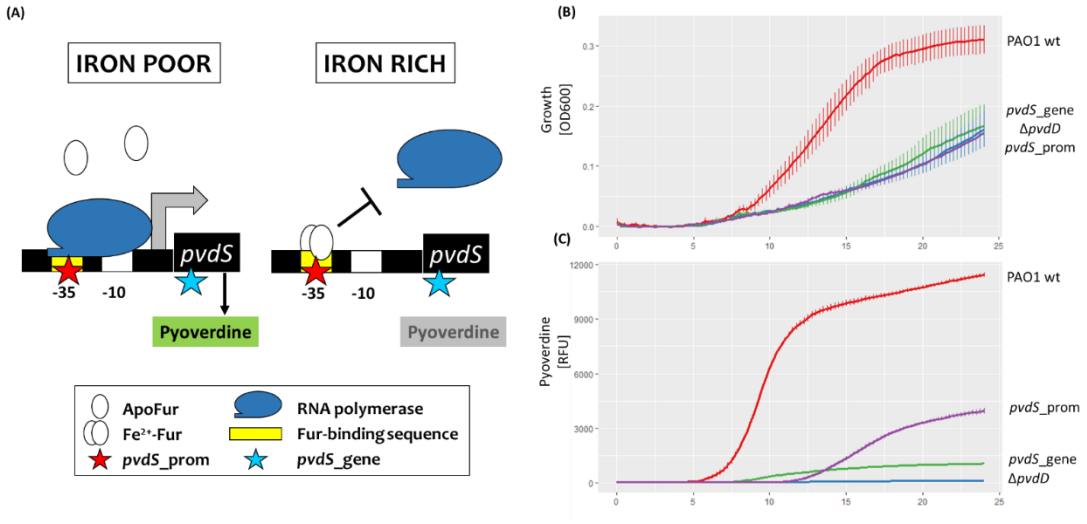

Fig. 1. Evolved clones of *P. aeruginosa* show impaired growth and pyoverdine production. (A) Schematic representation of *pvdS* regulation under iron-poor and iron-rich conditions. When iron is limited, *pvdS* is transcribed and upregulates pyoverdine biosynthesis. When iron levels in the cytoplasm are sufficient, Fur (ferric-uptake regulator) builds a complex with Fe^{2+}, which then binds to the *pvdS*-promoter site and inhibits transcription. Stars indicate SNPs in the mutant strains *pvdS*_prom (red) and *pvdS*_gene (blue). **(B+C)** A *P. aeruginosa* wildtype strain (PAO1 wt) and three different mutants with deficient pyoverdine production were grown in iron-limited media at 37 °C for 24 hours. Y axis shows **(B)** optical density measured at 600 nm or **(C)** pyoverdine-specific fluorescence (emission | excitation 400 nm | 460 nm). X axis shows time in hours. *ΔpvdD*: engineered knock-out mutant carrying an in-frame deletion of *pvdD*, encoding a part of the pyoverdine synthesis pathway. *pvdS*_gene: evolved mutant with single point mutation in *pvdS*, encoding the iron-starvation sigma factor PvdS. *pvdS*_prom: evolved mutant with a single point mutation in the promoter region of *pvdS*. Graph depicts means and standard errors based on four independent replicates per strain.

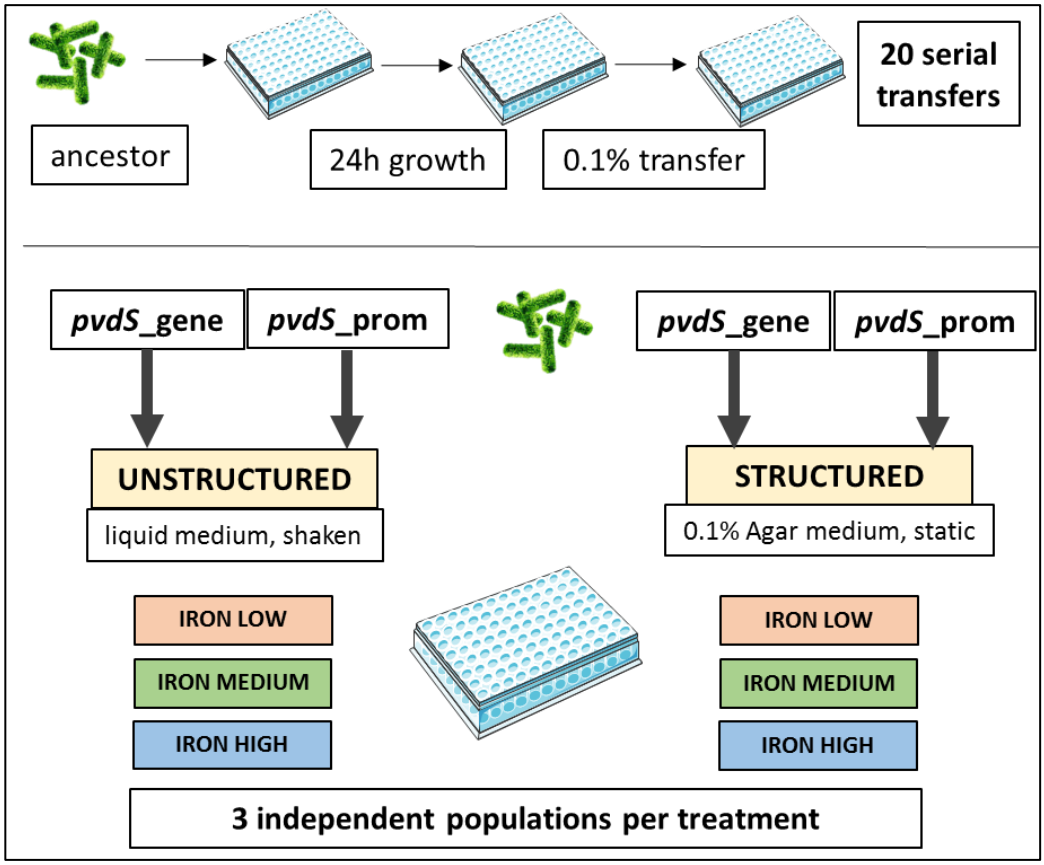

Fig. 2. Experimental evolution setup. Two mutant strains deficient in pyoverdine production, *pvdS*_gene and *pvdS*_prom, were allowed to evolve independently from each other and under different conditions. *pvdS* _gene carries a single point mutation in *pvdS*, encoding the iron-starvation sigma factor PvdS, while *pvdS*_prom carries a single point mutation in the promoter region of *pvdS*. The six environments used for experimental evolution differed both in their level of spatial structure (unstructured | structured) and in their iron content ("iron low": iron chelator only; "iron medium": iron chelator + 1 µM $FeCl_3$; "iron high": iron chelator + 40 µM $FeCl_3$). Each ancestral strain was serially transferred in each of the six media in threefold replication, resulting in a total number of 36 independently evolved populations. Image sources: Servier Medical Art (multiwell plate); depositphotos.com (bacteria).

Strain *pvdS*_prom carries a point mutation (G>T) in the consensus sequence of the -35 element in the promoter region upstream of *pvdS*. This mutant produces a wildtype sigma factor, but the transcription rate of PvdS is likely reduced.

Both of these mutations show strong defects in pyoverdine production and growth under iron-limited conditions (Fig. 1b+c). Pyoverdine production of the *pvdS*_gene strain was only 9.4 ± 0.1 % (mean ± SE) compared to the wildtype strain PAO1 (measured after 24 hours), and characterized by a low but steady production rate (Fig. 1c). While pyoverdine production was also reduced in *pvdS*_prom (34.7 ± 1.4 % relative to the ancestral wildtype strain), the production dynamic differed from *pvdS*_gene. The *pvdS*_prom strain had an extended phase, where no pyoverdine is produced, followed by a phase with a considerable production rate (Fig. 1c). Both mutant strains displayed substantial growth impairments, comparable to that of a constructed pyoverdine knockout (Fig. 1b). This indicates that the production of higher amounts of pyoverdine would be advantageous.

Further degradation and not re-evolution of pyoverdine production prevails

Following 20 days (approx. 200 generations) of experimental evolution in six different environments (2 different spatial structures x 3 different iron concentrations; Fig. 2), we screened 720 clones for their evolved levels of pyoverdine production and growth under iron limitation (Fig. 3). For each clone, we then calculated the per capita pyoverdine production (pyoverdine fluorescence divided by OD_{600}). Under the conditions of this assay, the ancestral strains *pvdS*_gene and *pvdS*_prom displayed 17.4 and 28.5 % (unstructured | structured), and 59.9 and 83.2 % (unstructured | structured) of the wildtype PAO1 pyoverdine production levels after 24 hours, respectively. Among the evolved clones, there were only very few (n = 5; 0.69%) that exhibited considerably increased

pyoverdine production levels (Fig. 3), indicating that reversion to higher levels of cooperation is rare. In contrast, we found a considerable number of clones (n = 29; 4.03 %) that showed either a complete abolishment or a further substantial reduction in pyoverdine production during evolution.

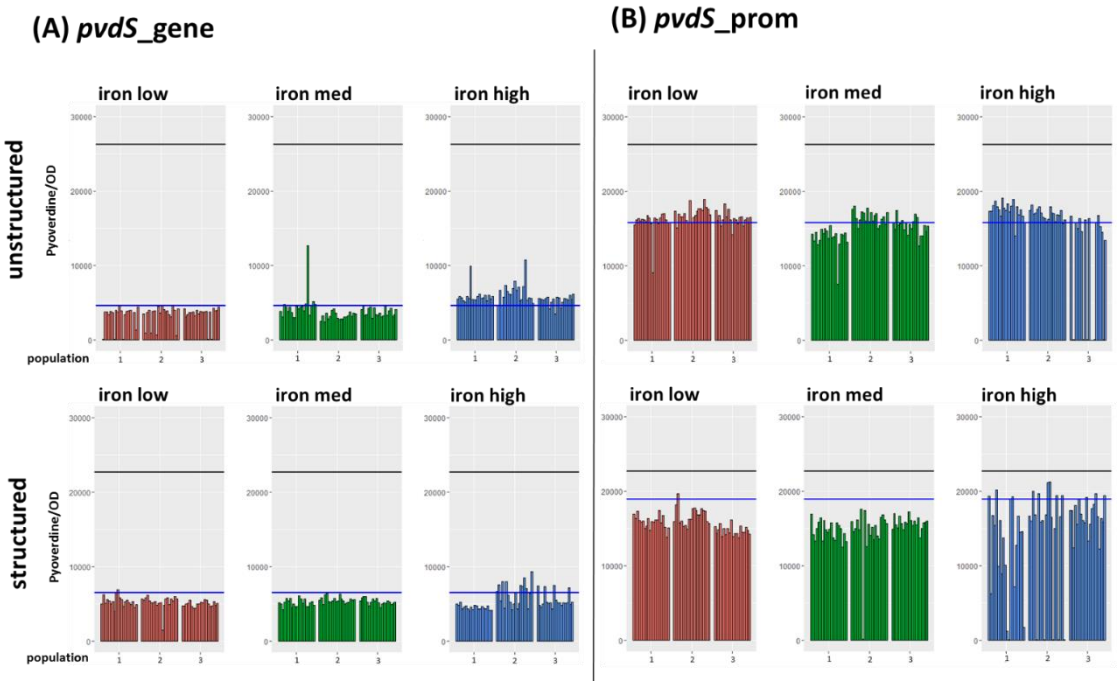

Fig. 3. Changes in pyoverdine production after experimental evolution in different environments. Two mutants with abnormal pyoverdine production were allowed to evolve in different media, and pyoverdine production under iron-limitation was subsequently measured in 720 evolved clones. Environments differed in their level of spatial structure (structured | unstructured) and in their iron content ("iron low": iron chelator only; "iron med": iron chelator + 1 µM $FeCl_3$; "iron high": iron chelator + 40 µM $FeCl_3$). **(A)** Clones evolved from the low-producer *pvdS*_gene, a mutant with a single point mutation in *pvdS*, encoding the iron-starvation sigma factor PvdS. **(B)** Clones evolved from the low-producer *pvdS*_prom, a mutant with a single point mutation in the promoter region of *pvdS*. Y axes show pyoverdine-specific fluorescence divided by growth (optical density at 600 nm) after 24 h of incubation. X axes show independent replicate populations the clones evolved in. Each bar represents a single measurement per evolved clone. The black line denotes the average wildtype production level in the same assay, while the blue line denotes the average production level of the respective low-producing ancestor.

There was an interaction between the genetic background and the environmental conditions under which these non- and extremely low pyoverdine-producing mutants appeared. In the *pvdS*_gene background, they appeared exclusively under low iron conditions, and were significantly more prevalent in unstructured compared to structured environments (Fisher's exact test, $p = 0.012$; Table 1). Since pyoverdine is important for growth under these iron-limited conditions, yet can be exploited in unstructured environments, this pattern suggests that the non- and extremely low pyoverdine-producing clones are cheats, which spread because they exploited the little amount of pyoverdine produced by *pvdS*_gene. In the *pvdS*_prom background, meanwhile, non- and extremely low-producers appeared almost exclusively under high iron conditions (Fisher's exact test, $p < 0.001$), but independently of the spatial structure (Fisher's exact test, $p = 0.78$; Table 1). This pattern indicates that pyoverdine production was most likely eroded due to disuse in iron-rich environments.

Table 1. Frequency of non- and low-producing strains per treatment

ancestor	*pvdS*_gene						*pvdS*_prom					
environment	structured			unstructured			structured			unstructured		
iron[a]	low	med	high	low	med	high	low	med	high	low	med	high
NLPs[b]	**1**	**0**	**0**	**11**	**0**	**0**	**0**	**1**	**9**	**0**	**0**	**7**
rest[c]	**59**	**60**	**60**	**49**	**60**	**60**	**60**	**59**	**51**	**60**	**60**	**53**

[a] low, medium (med) or high iron availability; see methods for details
[b] non- or low-producers, based on initial screening; corresponds to data shown in Fig. 3
[c] clones not in the NLP category

In-depth analysis of a subset of evolved clones confirms selection against pyoverdine

Since the large screen of 720 clones was based on a single replicate per clone (Fig. 3), we subjected the 34 clones with a putatively altered pyoverdine phenotype to a replicated in-depth phenotypic screen. We further included 23 clones with apparently unaltered pyoverdine phenotypes. For clones with the *pvdS*_gene background, we could confirm the phenotype of all clones that showed a further decrease in pyoverdine production (Fig. 4a). In fact, pyoverdine production was virtually absent in all of them. Conversely, we could only confirm the phenotype of two of the three mutants with putatively increased pyoverdine production, and even for the confirmed ones, the observed increase was marginal (Fig. 4b).

We obtained similar confirmation patterns for clones with the *pvdS*_prom background: confirmation rate was only high for clones with reduced but not for those with increased pyoverdine production levels (Fig. 4c+d). Finally, when examining the clones with a putatively unaltered pyoverdine, we found that 61 % (14 out of 23) of these clones indeed had a phenotype equal to their ancestral strain, whereas 35 % (8 out of 23) of the clones had pyoverdine production slightly but significantly reduced (Fig. S1). Taken together, these results confirm the patterns of our extensive screen (Fig. 3): there was selection to further reduce pyoverdine production, but no restoration of cooperation.

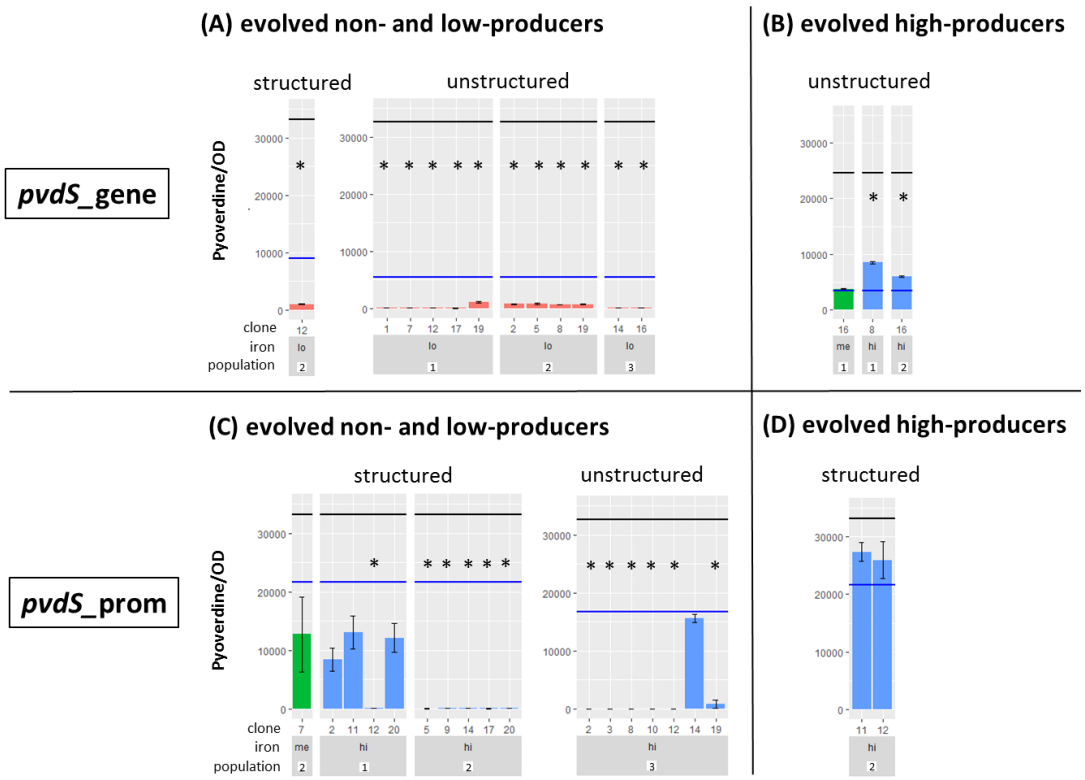

Fig. 4. Confirmed pyoverdine phenotypes in selected clones. Evolved clones with changes in pyoverdine production were re-tested to confirm their phenotype. Pyoverdine production was measured in iron-limited media. **(A)** Clones evolved from the low-producer *pvdS*_gene with low production levels in initial screening. **(B)** Clones evolved from the low-producer *pvdS*_gene with high production levels in initial screening. **(C)** Clones evolved from the low-producer *pvdS*_prom with low production levels in initial screening. **(D)** Clones evolved from the low-producer *pvdS*_prom with high production levels in initial screening. Y axes show pyoverdine-specific fluorescence divided by growth (optical density at 600 nm) after 24 h of incubation. X axes show independent replicate populations the clones evolved in and iron availability during experimental evolution. Bars represent mean values of three replicates per evolved clone. Error bars denote standard error of the mean. The black line represents the average wildtype production level in the same assay, while the blue line denotes the average production level of the respective low-producing ancestor. Bars are coloured by iron availability during evolution: red = low iron; green = medium iron; blue = high iron. We used one-way ANOVAs with Tukey's post-hoc test for comparisons relative to the low-producing ancestor. Asterisks indicate a significant difference ($p<0.05$) from the low-producing ancestor.

Evolved pyoverdine phenotypes are not based on further mutations in *pvdS*

We anticipated that both restoration and further reduction of pyoverdine production could be caused by reversion or compensatory mutations in the *pvdS* gene or its promoter. However, we found no support for this hypothesis when sequencing this genetic region for the subset of 57 clones described above (Table S1). All clones had retained the original, ancestral mutation inherited from their respective low-producing ancestor (SNP in the *pvdS* gene itself for *pvdS*_gene, SNP in the *pvdS* promoter region for *pvdS*_prom). Additionally, one clone from the *pvdS*_gene line gained an additional SNP in the *pvdS* promoter region, which however did not affect its phenotype. No additional mutations were found in any of the clones, indicating that the observed changes in pyoverdine production either represent an entirely phenotypic change, or are caused by mutations in regions other than *pvdS*.

DISCUSSION

Numerous studies used microbial systems to address a key question in evolutionary biology: how can cooperation be maintained in the face of cheats that exploit the cooperative acts performed by others [17–19]. Conversely, the question of what happens after a cheat has become fixed in the population has received much less attention. Would it be possible that cooperation re-evolves if environmental conditions and thus selection pressures change [20,21]? To tackle this question, we performed experimental evolution with *P. aeruginosa* cheat strains (mutants that produced greatly reduced amounts of the iron-scavenging public good pyoverdine), which had the potential to revert back to a full cooperative phenotype by a single point mutation. Despite this favourable genetic predisposition, we never observed reversion to cooperation, even under

conditions that had previously been identified as being favourable for cooperation. Instead, we observed the emergence of mutants that completely abolished pyoverdine production, and their frequency of appearance depended on both their genetic background and the environmental conditions. Taken together, our study highlights that the re-evolution of cooperation might be constrained in bacteria.

We can think of at least two reasons why there was no reversion from cheats back to cooperators. At the mechanistic level, it might be that the likelihood of acquiring a mutation that leads to reversion was simply too low. It is well known that mutations causing a loss of function are disproportionately more likely to occur than mutations resulting in a gain of function [22]. In the context of our experiment, re-evolution of pyoverdine production could have happened by a reversion to the ancestral PAO1 genotype (i.e. reversing the point mutation in the *pvdS* region) or by a compensatory mutation in *pvdS* or another regulatory element. Clearly, the number of mutational targets that would lead to reversion seem limited, and thus mutation supply might have been too low for revertants to arise.

At the ultimate level, it might be that we have not chosen the appropriate environmental conditions that would select for reversion. According to Hamilton's rule, we would expect selection for reverted cooperators when relatedness is relatively high and/or when the cost-to-benefit ratio of cooperation is relatively low. Although we have implemented experimental conditions promoting significant relatedness (through limited cell mixing in spatially structured environments) and reduced costs of pyoverdine production (at intermediate iron limitation), the chosen conditions were apparently not favourable enough to select for the re-evolution of cooperation. At first glance, this seems surprising because the chosen conditions have previously been shown to prevent the spreading of cheats and to maintain cooperation [8,10,23]. Our findings thus suggest

that the conditions for the evolution of cooperation are more stringent than those for the maintenance of cooperation. Indeed, social evolution theory predicts cooperation to be maintained when $rb = c$ (rare cheats cannot invade), while Hamilton's rule $rb > c$ must be met for cooperation to evolve. The fulfilment of this latter condition might require specific conditions (e.g. very high relatedness), as reverted cooperators would have to invade from extreme rarity, while being surrounded by clones exploiting any pyoverdine molecule diffusing away from the producer.

Instead of reversion to cooperation, we observed selection for mutants that further reduced or completely abolished pyoverdine production (Fig. 3+4). Intriguingly, the environments that promoted the spread of these mutants differed between *pvdS*_gene and *pvdS*_prom, indicating that different selection pressures can promote the same phenotype, and that the genetic background matters. For the *pvdS*_gene background, we found that the further degradation of pyoverdine production predominantly occurred with low spatial structure and under stringent iron limitation. As pyoverdine is important for growth under these conditions but widely shared due to mixing, we assume that these mutants spread because they cheated on the residual pyoverdine produced by the ancestral *pvdS*_gene. This finding confirms the notion that "cheating" is context-dependent, and shows that a strain that evolved as a cheat is still susceptible to further exploitation, despite its greatly reduced investment into a cooperative trait [24]. In contrast to this pattern, we observed the further degradation of pyoverdine production in the *pvdS*_prom background almost exclusively in iron-rich environments regardless of spatial structure. Because pyoverdine is not needed under iron-rich conditions, yet still expressed in low amounts [11,16], we assume that selection against pyoverdine production represents the erosion of an unnecessary trait.

We can only speculate about why the genetic background seems to matter for whether pyoverdine degradation is presumably driven by cheating or disuse. One possible explanation might reside in the different pyoverdine production profiles shown by the two strains. While *pvdS*_gene has a low but steady production rate, *pvdS*_prom delays pyoverdine production, but then produces pyoverdine at a higher rate compared to *pvdS*_gene. It could be that delaying the onset of pyoverdine production is a successful strategy to prevent the invasion of mutants with completely abolished pyoverdine production. With regard to trait erosion, it seems possible that *pvdS*_prom produces higher amounts of pyoverdine compared to *pvdS*_gene under iron-rich conditions; this would make this strain more susceptible for trait erosion because pyoverdine production is maladaptive under these conditions. Further studies are clearly needed to elucidate these pattern at both the proximate and ultimate level. The proximate level is of special interest here because the complete loss of pyoverdine production did not involve mutations in *pvdS*, which has been identified as the main target of selection for the initial reduction in pyoverdine production [7,12; Granato ET, Ziegenhain C & Kümmerli R, unpublished].

CONCLUSIONS

Our findings indicate that the evolution of cooperation through mutational reversion seems to be constrained. Reasons for this could be linked to the low number of mutational targets available that can lead to reversion, or the stringent selective conditions required to promote the spread of revertants. Clearly, the conditions that have previously been shown to maintain cooperation are not sufficient to promote the invasion of *de novo* re-evolved cooperators from rare. While we focussed on the re-evolution of cooperation via mutations, another alternative scenario

under natural conditions is that cheats may revert to cooperators through horizontal gene transfer [25,26]. This scenario has especially been advocated for cooperative traits located on plasmids [27,28]. While this is a plausible scenario for some social traits, it is unlikely to apply to siderophores, which are typically encoded on the chromosome. The insights gained from our study contribute to our understanding of the conditions necessary for a cooperative trait to evolve in microorganisms.

METHODS

Strains and growth conditions. We used *Pseudomonas aeruginosa* wildtype strain PAO1 (ATCC 15692) and a pyoverdine-negative mutant, both constitutively expressing GFP (PAO1-*gfp,* PAO1-Δ*pvdD-gfp*), as positive and negative controls for pyoverdine production, respectively. We further used PAO1-*pvdS*_gene and PAO1-*pvdS*_prom, two mutants with strongly reduced pyoverdine production, that evolved *de novo* from PAO1-*gfp* during experimental evolution in iron-limited media (2.5 gL^{-1} BactoPeptone, 3 gL^{-1} NaCl, 5 mgL^{-1} Cholesterol, 25 mM MES buffer pH = 6.0, 1mM MgSO4, 1mM $CaCl_2$, 200 µM 2,2'-Bipyridyl (Granato ET, Ziegenhain C & Kümmerli R, unpublished)). PAO1-*pvdS*_gene carries a non-synonymous point mutation (G>C) in the *pvdS* gene that leads to an amino acid change (Met135Ile). PAO1-*pvdS*_prom carries a point mutation (G>T) in the consensus sequence of the -35 element in the promoter region upstream of *pvdS*. Both mutants constitutively express GFP. Throughout this publication, the two mutants are referred to as "*pvdS*_gene" and "*pvdS*_prom".

For overnight pre-culturing, we used Luria Bertani (LB) medium, and incubated the bacteria under shaking conditions (190-200 rpm) for 16-18 hours. Optical density (OD) of pre-cultures was determined at a wavelength of 600 nm in a spectrophotometer. We induced strongly iron-

limiting growth conditions by using casamino acids (CAA) medium (5 gL^{-1} casamino acids; 1.18 gL^{-1} $K_2HPO_4 \cdot 3H_2O$; 0.25 gL^{-1} $MgSO_4 \cdot 7H_2O$) supplemented with 25 mM HEPES and 400 µM of the iron chelator 2,2'-Bipyridyl. For conditions with medium or high iron availability, we further added $FeCl_3$ at final concentrations of 1 µM or 40 µM, respectively. We manipulated the spatial structure of the environment by growing bacteria either in liquid medium under shaking conditions (180 rpm; unstructured environment) or in viscous medium containing 0.1% agar under static conditions (structured environment). All experiments in this study were conducted at 37°C. All chemicals were purchased from Sigma-Aldrich, Switzerland.

Ancestral growth and pyoverdine kinetics. To measure growth and pyoverdine production kinetics of all strains in iron-limited media prior to experimental evolution, we washed bacterial pre-cultures twice with sterile NaCl (0.85%), adjusted OD_{600} to 1.0, and diluted 10^{-4} into 200 µL of iron-limited CAA (Bipyridyl 400 µM) per well in a 96-well plate. The plate was then incubated in a Tecan Infinite M-200 plate reader (Tecan Group Ltd., Switzerland) for 24 hours, and OD_{600} and pyoverdine-specific fluorescence (emission 400 nm, excitation 460 nm) were measured every 15 minutes.

Experimental evolution. We conducted experimental evolution with *pvdS*_gene and *pvdS*_prom as starting points. We let each strain evolve independently under six different experimental treatments in a full-factorial design: 2 spatial structures (unstructured vs. structured) x 3 iron availabilities (low vs. medium vs. high iron availability) in three replicate independent lines (Fig. 2). At the start of the experimental evolution, overnight cultures of both clones were washed twice with NaCl (0.85%), adjusted to an OD_{600} of 1.0 and diluted 1:1000 into 200 µL of nutrient medium in 96-well plates. Plates were wrapped with parafilm, incubated for 24 hours and subsequently diluted 1:1000 in fresh nutrient medium. We

repeated this cycle for 20 consecutive transfers, allowing for approximately 200 generations of bacterial evolution (Fig. 2). At the end of the experiment, we prepared freezer stocks for each evolved population (n = 36) by mixing 100 µL of bacterial culture with 100 µL of sterile glycerol (85%). Samples were stored at -80°C.

Isolation of single clones. To check whether evolved clones showed altered pyoverdine production levels compared to the ancestral *pvdS*_gene and *pvdS*_prom strains, we isolated a total of 720 evolved clones (20 per replicate and treatment). Specifically, we regrew evolved bacterial populations from freezer stocks in 5 mL LB medium for 16-18 hours (180 rpm) and subsequently adjusted them to OD_{600} = 1.0. Then, 200 µL of 10^{-6} and 10^{-7} dilutions were spread on large LB agar plates (diameter 150 mm), which we incubated at 37°C for 18-20 hours. We then randomly picked twenty colonies for each of the 36 evolved populations, and immediately processed the clones for the pyoverdine measurement assay (see below).

Screen for evolved pyoverdine production levels. For each of the 720 evolved clones, we transferred a small amount of material from the agar plate directly into 200 µL of CAA + Bipyridyl (400 µM) in individual wells on a 96-well plate. We incubated plates with clones originating either from unstructured environments or structured environments for 24 hours under shaken (180 rpm) or static conditions, respectively. Following incubation, we measured OD_{600} and pyoverdine-specific fluorescence (emission 400 nm, excitation 460 nm) in the Tecan Infinite M-200 plate reader as a single endpoint measurement. As controls, we included in three-fold replication on each plate: the high-producing PAO1 wildtype (positive control); the pyoverdine knockout mutant PAO1-Δ*pvdD-gfp* (negative control); the two low-producing mutants *pvdS*_gene and *pvdS*_prom; and blank growth medium. To preserve all tested clones for future experiments, we

mixed 100 µL of bacterial culture with 100 µL of sterile glycerol (85%) for storage at -80°C.

Confirmation of evolved pyoverdine phenotypes. Based on the screen above, we identified 34 clones with an altered pyoverdine production level (Table S1). Specifically, we found five clones that seem to have restored pyoverdine production by roughly 50% (i.e. in terms of the difference between the low-producing ancestor cheat and the high-producing wildtype) and 29 clones that seem to produce less than 33% of pyoverdine compared to their ancestral pyoverdine low-producers (either *pvdS*_gene or *pvdS*_prom). We subjected these clones to an in-depth repeated screening of their pyoverdine phenotype. In addition, we selected two random clones per treatment (n = 24), from different evolved populations, that displayed no change in their production levels (compared to *pvdS*_gene or *pvdS*_prom). One clone had to be excluded due to contamination, so that the final sample size for this group of clones was n = 23. For all of these evolved clones (n = 57), we re-measured their pyoverdine production level in three-fold replication using the same protocol and controls as described above.

Sequencing of *pvdS* promoter and coding region. Since the ancestral low-producing strains (*pvdS*_gene or *pvdS*_prom) had mutations in the *pvdS* gene or its promoter, we were wondering whether the altered phenotypes observed in the evolved clones were based on reversion or additional mutations in this genetic region. To address this question, we PCR amplified and sequenced the *pvdS* gene and the upstream region containing the promoter sequence of all 57 evolved clones screened above. PCR mixtures consisted of 2 µl of a 10 µM solution of each primer, *pvdS_fw* (5'-GACGCATGACTGCAACATT-3') and *pvdS_rev* (5'-CCTTCGATTTTCGCCACA-3'), 25 µl Quick-Load Taq 2X Master Mix (New England Biolabs), 1 µl of DMSO, and 20 µl of sterile Milli-Q water. We added bacterial biomass from glycerol stocks to the PCR mixture distributed in

96-well PCR plates. Plates were sealed with an adhesive film. We used the following PCR conditions: denaturation at 95°C for 10 min; 30 cycles of amplification (1 min denaturation at 95°C, 1 min primer annealing at 56°C, and 1 min primer extension at 72°C); final elongation at 72 °C for 5 min. The PCR products were purified and commercially sequenced using the *pvdS_fw* primer. While sequencing worked well for 51 clones, it failed for two clones, and resulted in partial sequences for six clones (Table S1).

Statistical Analysis. All statistical analyses were performed using R 3.2.2 [29]. We tested for treatment differences in the frequency of non- or low-producing strains using Fisher's exact test and corrected for multiple testing using the Bonferroni correction. To compare pyoverdine production of evolved clones to that of the low-producing ancestors, we used one-way analyses of variance (ANOVA) and corrected for multiple testing using Tukey's HSD (honest significant difference) test.

Authors' contributions. EG and RK planned the experiments. EG carried out the experiments and conducted statistical analysis. EG and RK analyzed and interpreted the data, and wrote the manuscript.

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7.2 Supporting Material

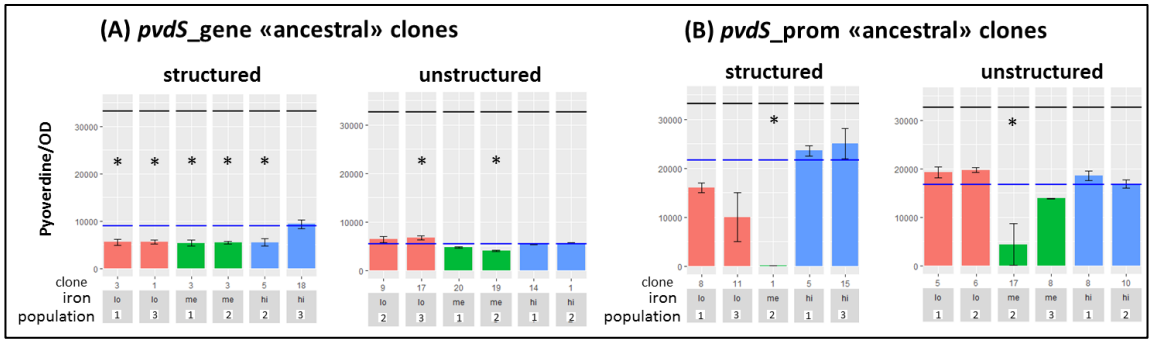

Fig. S1. Confirmed pyoverdine phenotypes in selected clones. Evolved clones with ancestral pyoverdine production levels were re-tested to confirm their phenotype. Pyoverdine production was measured in iron-limited media. **(A)** Clones evolved from the low-producer *pvdS*_gene. **(B)** Clones evolved from the low-producer *pvdS*_prom. Y axes show pyoverdine-specific fluorescence divided by growth (optical density at 600 nm) after 24 h of incubation. X axes show independent replicate populations the clones evolved in and iron availability during experimental evolution. Bars represent mean values of three replicates per evolved clone. Error bars denote standard error of the mean. The black line represents the average wildtype production level in the same assay, while the blue line denotes the average production level of the respective low-producing ancestor. Bars are coloured by iron availability during evolution: red = low iron; green = medium iron; blue = high iron. We used one-way ANOVAs with Tukey's post-hoc test for comparisons relative to the low-producing ancestor. Asterisks indicate a significant difference ($p<0.05$) from the ancestor.

Table S1. Clones selected for in-depth analysis and sequencing

Population[1]	clone #	pyoverdine phenotype[2]	*pvdS* mutation[3]	sequence length [bp][4]	*pvdS* status[5]	comment[6]
78_s_hi_2	5	ancestral	2722579 G>C	778	ancestral	
78_s_hi_3	18	ancestral	2722579 G>C; 2722596 A-->C	778	mutated	additional SNP in promoter region
78_s_lo_1	3	ancestral	2722579 G>C	778	ancestral	
78_s_lo_2	12	low	2722579 G>C	778	ancestral	
78_s_lo_3	1	ancestral	2722579 G>C	778	ancestral	
78_s_me_1	3	ancestral	2722579 G>C	778	ancestral	
78_s_me_2	3	ancestral	2722579 G>C	778	ancestral	
78_u_hi_1	14	ancestral	2722579 G>C	778	ancestral	
78_u_hi_1	8	high	2722579 G>C	778	ancestral	
78_u_hi_2	1	ancestral	2722579 G>C	778	ancestral	
78_u_hi_2	16	high	2722579 G>C	778	ancestral	
78_u_lo_1	1	low	2722579 G>C	778	ancestral	
78_u_lo_1	7	low	2722579 G>C	778	ancestral	
78_u_lo_1	12	low	2722579 G>C	778	ancestral	
78_u_lo_1	17	low	2722579 G>C	778	ancestral	
78_u_lo_1	19	low	2722579 G>C	778	ancestral	
78_u_lo_2	2	low	2722579 G>C	778	ancestral	
78_u_lo_2	5	low	N/A	N/A	N/A	sequencing failed
78_u_lo_2	8	low	2722579 G>C	778	ancestral	
78_u_lo_2	19	low	2722579 G>C	778	ancestral	
78_u_lo_2	9	ancestral	2722579 G>C	778	ancestral	
78_u_lo_3	14	low	2722579 G>C	778	ancestral	
78_u_lo_3	16	low	2722579 G>C	778	ancestral	
78_u_lo_3	17	ancestral	2722579 G>C	778	ancestral	
78_u_me_1	20	ancestral	2722579 G>C	778	ancestral	
78_u_me_1	16	high	2722579 G>C	778	ancestral	
78_u_me_2	19	ancestral	2722579 G>C	778	ancestral	
97_s_hi_1	2	low	2722079 G>T	778	ancestral	
97_s_hi_1	11	low	2722079 G>T	778	ancestral	
97_s_hi_1	12	low	2722079 G>T	778	ancestral	
97_s_hi_1	20	low	2722079 G>T	778	ancestral	
97_s_hi_1	5	ancestral	2722079 G>T	778	ancestral	
97_s_hi_2	11	high	2722079 G>T	778	ancestral	
97_s_hi_2	12	high	2722079 G>T	778	ancestral	
97_s_hi_2	5	low	2722079 G>T	778	ancestral	
97_s_hi_2	9	low	2722079 G>T	778	ancestral	
97_s_hi_2	14	low	2722079 G>T	778	ancestral	
97_s_hi_2	17	low	2722079 G>T	628	ancestral	incomplete sequence (-150 bp)
97_s_hi_2	20	low	2722079 G>T	778	ancestral	
97_s_hi_3	15	ancestral	2722079 G>T	778	ancestral	
97_s_lo_1	8	ancestral	2722079 G>T	778	ancestral	
97_s_lo_3	11	ancestral	2722079 G>T	778	ancestral	

97_s_me_2	7	low	2722079 G>T	778	ancestral	
97_s_me_2	1	ancestral	2722079 G>T	778	ancestral	
97_u_hi_1	8	ancestral	2722079 G>T	778	ancestral	
97_u_hi_2	10	ancestral	2722079 G>T	683	ancestral	incomplete sequence (-95 bp)
97_u_hi_3	2	low	2722079 G>T	778	ancestral	
97_u_hi_3	3	low	2722079 G>T	778	ancestral	
97_u_hi_3	8	low	2722079 G>T	642	ancestral	incomplete sequence (-136 bp)
97_u_hi_3	10	low	2722079 G>T	639	ancestral	incomplete sequence (-139 bp)
97_u_hi_3	12	low	2722079 G>T	639	ancestral	incomplete sequence (-136 bp)
97_u_hi_3	14	low	2722079 G>T	778	ancestral	
97_u_hi_3	19	low	2722079 G>T	778	ancestral	
97_u_lo_1	5	ancestral	2722079 G>T	628	ancestral	incomplete sequence (-150 bp)
97_u_lo_2	6	ancestral	2722079 G>T	778	ancestral	
97_u_me_2	17	ancestral	N/A	N/A	N/A	sequencing failed
97_u_me_3	8	ancestral	2722079 G>T	778	ancestral	

[1] 78 = *pvdS*_gene, 97 = *pvdS*_prom; s = structured; u= unstructured; lo = iron low; me = iron medium; hi = iron high
[2] pyoverdine phenotype compared to the low-producing ancestor in 1st screen (Fig. 3)
[3] genome location in PAO1 reference genome (*pseudomonas.com*) and respective change in nucleotide
[4] length of sequenced promoter region and *pvdS* gene; full sequence length = 778 bp (only *pvdS* gene: 564 bp)
[5] "ancestral": clone harbors only the mutation already present in the low-producing ancestor
"mutated": clone harbors mutation in addition to the one already present in the low-producing ancestor
[6] in case of incomplete sequencing, numbers in brackets indicate number of base pairs missing from the end of the *pvdS* gene

8 CONCLUDING DISCUSSION

"All you really need to know for the moment is that
the universe is a lot more complicated than you might think,
even if you start from a position of thinking it's pretty
damn complicated in the first place."

~ Douglas Adams, The Hitchhiker's Guide to the Galaxy (1979)

8.1 Synopsis

Bacteria are highly social organisms and engage in several cooperative behaviors [5]. Secreted, shareable metabolites, so called "public goods", are important cooperative traits in the opportunistic pathogen *Pseudomonas aeruginosa* [72,74]. During an infection, *P. aeruginosa* relies on several secreted virulence factors to colonize the host and damage the host tissue [106,112,115]. In this thesis, I analyzed the role of compound secretion in *P. aeruginosa* in the context of cooperation and infection.

In Project 1, I investigated how an important cooperative trait affects the virulence of *P. aeruginosa*. The siderophore pyoverdine is cooperatively secreted by *P. aeruginosa* cells during infections in order to scavenge for essential iron in the host. While it was known that pyoverdine is important in some infection contexts, it was unclear whether its effect varies between study systems. Using published data from 81 infection experiments, I conducted a meta-analysis on the relationship between pyoverdine production of the infecting *P. aeruginosa* strain, and host survival. My analysis included data from experiments with different host species and bacterial strains. I found that pyoverdine production was consistently involved with virulence across different infection contexts, but the magnitude of the effect varied considerably. In many cases, the effect of pyoverdine was quite minor. This suggests that pyoverdine production is important, but not indispensable in infections.

My findings in Project 1 also reinforce the notion that virulence in a given pathogen is a highly context-dependent and complex trait. To understand how virulence evolves in bacterial pathogens, a more holistic approach is needed. This is especially true for generalist, opportunistic pathogens that occur and evolve in many different environments. Therefore, I continued my investigation of virulence in *P. aeruginosa* in Project 2 and looked at pathogenicity and virulence factor production from a more ecological perspective.

My goal was to study how *P. aeruginosa*, an opportunistic pathogen, adapts to conditions inside and outside of a host organism, and how adaptation would affect virulence towards the same host. I let wild type *P. aeruginosa* evolve with and without a host, the nematode *Caenorhabditis elegans*, while also varying the degree of spatial structure in the environments. I found that virulence dropped dramatically in unstructured environments, and that this was mainly driven by two forces: accidental effects, where bacteria lost virulence traits not needed outside of the host; and social effects, when mutants lost the ability to produce pyoverdine, a shareable virulence factor, and displaced the wild type through social exploitation.

This exploitation of wild type - i.e. producing - cells by non-producing "cheat" mutants is common for cooperative traits in bacteria. Pyoverdine secretion in *P. aeruginosa* is one of the best-known study systems in this context, and several studies have described the success of cheats in different environments [20,66,67,177]. Until now, however, it was not clear what happens after a cheat has invaded a population of producers.

In Project 3, I aimed to fill this gap by examining whether clones that have become deficient for pyoverdine production can re-evolve cooperation under certain conditions, or whether the trait is degraded even further. I let two such clones evolve under different degrees of iron availability and spatial structure, and measured changes in pyoverdine production. I did not observe evolution towards more cooperation, but instead pyoverdine production was further reduced under conditions where it was useful (low iron availability), suggesting continued exploitation of the cooperative trait. Furthermore, I found that pyoverdine production was eroded under iron-rich conditions, an effect which can likely be attributed to erosion of an unnecessary trait. Thus, my findings show that the conditions for the evolution of cooperation might be more stringent than previously thought.

While the individual results for each project are discussed in detail in the individual chapters, I will here present the implications of my findings in a wider context. I will start by discussing what constitutes a virulence factor, followed by a section on the evolution of opportunistic pathogens, and a general outlook on social evolution studies in bacteria.

8.2 What is a "virulence factor"?

In Project 1, I concluded that pyoverdine plays a role in *P. aeruginosa* infections, but it is not essential. In many experiments, mutants deficient for pyoverdine were still able to infect and kill a variety of host organisms, albeit more slowly. In a few instances, pyoverdine was even completely dispensable. Given this high variability in its importance for infections, can pyoverdine still be considered a general "virulence factor"? How do we define a "virulence factor"?

Virulence is often defined as the degree of pathogen-induced host damage [178]. As such, it can be used as a proxy for disease severity on a spectrum from "lower" to "higher". Virulence can then be contrasted to the *pathogenicity* of an organism, which is a more qualitative assessment of its potential to cause disease [179]. An alternative definition of virulence describes it as "the ability to enter, replicate, and persist in a host", with no mention of adverse effects on the host [180]. I disagree with this particular definition, as this would classify all commensal bacteria as "virulent", which is clearly not very useful. From an evolutionary point of view, virulence can be expressed as a pathogen-induced loss of fitness in the host [135]. Throughout this thesis, I used a relatively broad definition of virulence: damage and/or increased host mortality due to infection.

Virulence factors (VF) are commonly defined as any property of a pathogen that enables it to infect a host. This includes traits that facilitate colonization of the host, damage the host tissue, or evade or suppress the

immune system of the host [181–184]. In order to rule out traits essential to general cell viability, genes necessary for growth in a nutrient-rich broth are usually excluded. When trying to distinguish between a VF and a non-VF, problems can arise i) when a trait is involved in the infection process, but not essential to it; ii) when their importance for the infection process varies considerably between infection types; or iii) when they carry out an important function outside of the host.

Pyoverdine in *P. aeruginosa* is a good example for a trait that can be used to explore these "grey areas" of VF definitions. In Project 1, I demonstrated that pyoverdine is important for virulence in many different hosts, but pyoverdine mutants were often still able to cause considerable damage to the host. This suggests that this trait provides a benefit to the pathogen during infections, but is not strictly necessary. I also found that the importance of pyoverdine for virulence varied greatly among host species. This begs the questions: what if a trait is essential for virulence in one host species, but has no purpose in another? Would it still be considered a general VF? Importantly, the role of the VF can also change over the course of a single infection. For example, the switch from acute to chronic infections triggers physiological changes both in the host and in the pathogen [185–187]. A VF might then only be relevant at a certain point in time during an infection. The solution here is to always provide the relevant context, such as host species, when describing the role of a VF.

Pyoverdine can also be used to illustrate the fact that VFs can be important in different contexts. It is not only secreted to scavenge iron within the host, but also in natural, non-host environments [20,79]. Similarly, other virulence factors, such as proteases, are likely to confer benefits outside of the host context, thus contributing to the metabolic versatility in opportunistic pathogens. I have also addressed this in Project 2, where I found that selection acts on virulence factors both inside and outside of the host.

To sum up, it comes as no surprise that a phenomenon as complex as virulence is influenced by multiple factors, both on the pathogen and on the host side. As a result, it is nearly impossible to fit bacterial traits into a binary classification of VFs versus non-VFs. A lot of ambiguity can be avoided by i) conducting careful phenotypic assays that screen for damage to the host, and ii) providing sufficient context for the classification of a focal trait as a VF, such as host organism and infection type. However, several studies have identified potential pathogens and virulence traits purely by DNA sequence analysis, an approach that has gathered some amount of criticism [188,189]. In my opinion, this can easily lead to an overly simplified view on virulence and ignores much of what we know about pathogenicity.

Screening for virulence phenotypes can be cumbersome, as it involves the generation of large mutant libraries to screen for genes predictive of damage to the host [190,191]. But these steps are necessary if we want to integrate the whole infection context (host species, infection type) in our classification of VFs. A meta-analysis, as I conducted in Project 1, can greatly facilitate this by compiling data from different phenotypic virulence experiments.

8.3 *Quo vadis*, opportunistic pathogens?

While the previous section focused on mechanistic aspects of virulence and virulence factors, I will elaborate here on how virulence evolves in bacterial pathogens.

Understanding the evolution of bacterial pathogens is crucial in the search for better therapeutic interventions and ways to prevent epidemics. Among the most important properties to analyze in any pathogen are i) which organisms it can infect, ii) its transmissibility, and iii) how severe the symptoms are once the host is infected. Since the vast majority of microorganisms do not cause disease in plants or animals, we

can ask why some bacteria are virulent at all. After all, they are harming the very host they are exploiting for nutrients. A popular hypothesis states that harm to the host, i.e. virulence, is an unavoidable byproduct of growth within a host and transmission to a new host. A virulent trait is then maintained by natural selection through a trade-off between the benefits of transmission to the next host, and the costs of host mortality [126,127].

Despite the majority of pathogenic bacteria being generalists and opportunists (see introduction section 4.2.2), much of the theory on the evolution of virulence focuses on obligate pathogens. But unlike obligates, opportunists spend most of their existence living freely in the environment, and how this outside-host selection differs from within-host selection has received very little attention [129,192]. I added a piece to the puzzle in Project 2, by conducting experimental evolution with an opportunistic pathogen to disentangle selective pressures in both the host- and the non-host environment. My findings relate to current virulence evolution theory in two important aspects.

First, I found evidence for virulence being affected by purely environmental adaptation. When *P. aeruginosa* evolved without nematodes, virulence factors were under selection and their expression levels changed over time. When the evolved bacteria encountered their host later on, they exhibited a certain level of virulence that was not necessarily adaptive, but simply a random byproduct of selection in the non-host environment. This effect, named "accidental virulence", had been suggested before [129,193] and I found empirical evidence for it in my evolution experiment.

Second, I showed that decreased spatial structure leads to the evolution of lower virulence, a finding that diametrically opposes findings in viruses [137,138]. These opposite findings are likely due to differences in how virulence is realized. While viruses "hijack" and manipulate the cellular machinery of the host cells for their replication and transmission, virulence in opportunistic bacteria is often mediated by secreted, secondary

metabolites, which are subject to social interactions [16,72,103]. Here, I demonstrated that low spatial structure can then support the invasion of non-producing clones, effectively lowering virulence (Project 2).

Many unique aspects of virulence evolution in opportunistic pathogens are rooted in the fact that the virulence factors they secrete i) often also perform functions outside of the host environment and are not "specially tailored" to the host; and ii) can be subject to social interactions such as cheating. Neither of these scenarios are well-represented in most theoretical frameworks of virulence evolution, which often assume single, specialized pathogens infecting a single host. For one of the main virulence factors in *P. aeruginosa*, pyoverdine, I showed i) in Project 1 that it is important but not essential for infections, supporting the notion of a non-specialized metabolic process used in many environments, and ii) in Project 2 I demonstrated that it is indeed under social selection outside of the host. These findings strongly suggest that virulence factors are under selection within and outside of the host, and are subject to social dynamics.

In recent years, new theoretical frameworks for virulence evolution have started to incorporated these aspects [136,194], but they are still in the minority. A comprehensive review on virulence evolution from 2016 explicitly mentions opportunistic pathogens only once, citing two papers, and only features a short section on cooperation in pathogens [195]. This leads me to conclude that more research is still needed to adequately capture opportunistic pathogens, and to understand the relative social effects of different virulence factors and their influence on virulence evolution.

This is especially pressing in light of recent efforts to "disarm" pathogens by targeting virulence factors. These so-called "anti-virulence treatments" aim to reduce pathogen virulence without affecting essential cell functions [196–198]. By disarming pathogens rather than killing them, they could potentially avoid the selection pressure regular antibiotics impose,

thus reducing selection for resistance. More research on opportunistic pathogens and the sociality of virulence factors, combined with meta-analytical comparisons (such as Project 1) could thus inform us on which traits would be best suited as potential targets for anti-virulence therapies.

8.4 Rethinking the evolution of cooperation

Cooperation is widespread in nature and facilitates many exciting biological phenomena, such as group living in higher animals, cross-kingdom symbioses, and multicellularity [199–201]. Despite its prevalence, cooperation poses a significant challenge to evolutionary biology, because it opposes the traditional view of "*Nature, red in tooth and claw*"[1], where individuals compete and constantly try to maximize their own reproductive fitness. How cooperation has evolved is therefore of significant interest, and many studies have attempted at defining a set of circumstances needed for a cooperative trait to develop [202–204]. Bacteria have become popular study systems to address these questions, because we can rationally engineer their environments and follow evolution in real-time due to their rapid reproduction.

We know that cooperation, once it is in place, can be destabilized through the evolution of non-cooperative cheats (see introduction section 4.1.3). Factors that prevent this destabilization could potentially also facilitate the evolution of cooperation. However, despite the conceptual similarities between cheat prevention and evolution of cooperation, these two situations are not identical. I will now discuss the differences between these two scenarios in the light of my findings in Project 3.

[1] excerpt from: Alfred Lord Tennyson, *In Memoriam A. H. H.* (1850)

There, I evolved two pyoverdine cheats in different environments to test whether they could re-evolve into full cooperators. These cheat mutants were not fully deficient for pyoverdine, but instead displayed very low production levels. When I let these two mutants evolve in monoculture, I observed the evolution of secondary cheats, who had even lower pyoverdine production levels than the original cheats. While these secondary cheats arose with low spatial structure, their invasion was impeded with high spatial structure. This result is in line with other empirical studies [67,80] showing that high spatial structure leads to increased privatization of public goods and high relatedness among the cells, which both make it hard for *de novo* cheats to exploit the remaining producers.

On the other hand, the structure I imposed did not lead to the widespread re-evolution of pyoverdine high-producers. This demonstrates that the absence of selection for cheats does not necessarily lead to the selection of more cooperative clones. One possible explanation for this state of equilibrium, where neither cheats nor cooperators are strongly selected for, could be derived from Hamilton's rule (introduced in section 4.1.3). When $r*B \approx C$, the costs C of cooperation (pyoverdine production) are directly offset by its benefit B scaled by relatedness r. The relatedness imposed in my "high spatial structure" treatments seems to lead to such an equilibrium state. The low-cooperation-strategy of the original cheats is then evolutionary robust, in the sense that it cannot be outcompeted by clones with more - or less - cooperation.

I also found that secondary cheats readily evolved under conditions of low iron availability. Pyoverdine production is high, and also returns the highest benefits when iron is scarce, suggesting that the cooperative trait is highly "exploitable" there, which facilitates cheat selection. Yet again, low iron availability did not lead to the evolution of pyoverdine high-producers, despite the large fitness benefits that could have been gained. This suggests that the cost of being further exploited was higher than the benefits of higher production levels.

Here, we have to point out a crucial difference between "limiting cheats invasion" and the evolution of cooperation "from scratch": when a cooperative trait evolves in the first place, it is possible that it starts as a more "private" trait that is not perfectly and efficiently exploitable by surrounding non-cooperators. In my setup, a reverted cooperator would be surrounded by clones that have the correct receptor for uptake and which had evolved *precisely because* they were good at exploiting.

It is also possible that evolved clones upregulated their production of their secondary siderophore in response to selection pressure. This points to another difference to the origins of cooperation: here, the siderophore trait is already so established, that bacteria have alternative mechanisms at their disposal which they can switch to if needed. The exploitable trait can then be bypassed, thus preventing efficient exploitation by cheats. But this is likely not applicable for the original evolution of cooperation.

To conclude, I propose that i) care needs to be taken when transferring results on the maintenance of cooperation to the evolutionary origins of cooperation; and ii) the conditions needed for the evolution of cooperation are more stringent than previously thought, at least for microbial cooperation based on secreted public goods. Coming back to Hamilton's rule: for a cooperative trait to be selected for, r*B > C has to be fulfilled. In Project 3, I aimed at providing large benefits B for cooperation (by making iron very scarce) and increasing relatedness r (by ensuring high spatial structure). Still, cooperation was not selected for.

But maybe more stringent conditions are needed. Relatedness (implemented through spatial structure) could be increased even more, for example by growing bacteria on a completely solid surface. Iron limitation could also be exacerbated: in my experiment, pyoverdine low-producing cheats were still able to divide and survive, albeit worse than the ancestral cooperators. Iron-starving evolving cells even further would probably boost the benefits B of cooperation. One could of course engineer an extreme environment where cheats alone are not able to

grow at all, thereby maximizing the benefits of increased pyoverdine production, but then the initial absence of growth would stop mutation supply and prevent natural selection. It is thus entirely possible that the parameter space for the selection of cooperation is very narrow, and can only be captured under very stringent conditions with even higher spatial structuring and less iron.

9 PERSPECTIVES

In the following chapter, I would like to use the opportunity to provide the historical context for the questions I strove to answer, and discuss the importance of studying microbial interactions in general.

This section is meant to attract a layperson's interest in the topics I explored in this thesis, and as such it is written as a "popular science" piece without in-text citations.

9.1 No Microbe is an Island

> "The 31st of May, I perceived in the same water more of those Animals […] And I imagine, that [ten hundred thousand] of these little Creatures do not equal an ordinary grain of Sand in bigness: And comparing them with a Cheese-mite (which may be seen to move with the naked eye) I make the proportion of one of these small Water-creatures to a Cheese-mite, to be like that of a Bee to a Horse […]."
>
> ~Antonie van Leeuwenhoek (1632-1723)

One afternoon in the summer of 1675, Antonie van Leeuwenhoek, a lens maker in the Dutch trading town of Delft, glanced into his newest hand-made microscope and made a discovery for the ages. Earlier that day, he had had the whimsical idea of investigating a drop of water, collected from a flowerpot outside of his house. He was immediately struck by the amazing diversity of minuscule creatures whizzing about, and became the first human to ever see a microbe. Over the next decades, he continued his observations and documented his endeavors in great detail. Despite some early attention his findings received from the scholars of his time, the interest in his "little Creatures" waned again after his death. In the 1730s, when Carl Linnaeus started to classify all life, he lumped all microbes into the genus *Chaos*, meaning "formless", and another 150 years would pass before the next earnest efforts to study microbial life. Things only really changed in the mid-nineteenth century, largely thanks to a French chemist named Louis Pasteur and the German physician Robert Koch who demonstrated that bacteria can cause disease.

When scientists started exploring the world of microbes for the first time, nobody could have imagined that entire ecosystems were waiting to be discovered. At a time when all continents had been explored, and all final

frontiers were thought to be conquered, people slowly started to realize that there was an entire universe to be examined, invisible to us, covered by the "veil of scale". For most of our history, this universe was simply too small to be noticed. We have come a long way since Linnaeus, and now understand how essential microbes are to the history of our planet, to us, and to most other forms of life on earth. Thanks to modern microscopy technology and advances in culturing techniques, we now have the possibility to tap into this universe of creatures and explore their lives and their interactions with each other.

The microbiologists of our time have a great task at hand: to step into the lens maker's footsteps and investigate the microbial "jungle"; to find out who is the predator and who is the prey; to learn which microbes help their neighbors, and who are the ones that kill others on sight; to discover symbiotic relationships where none can live without the other, and to understand their language.

Out of all these different and exciting behaviors animals and microbes engage in, cooperation has always been an especially fascinating topic for scientists, especially for those interested in evolution. At the surface, it does seem counter-intuitive: if everything is selfishly struggling for survival, why should any organism invest in others? This even caused Darwin to cast a shadow of a doubt on his own theory of evolution: he famously called altruism, an extreme form of cooperation, a "special difficulty" and even "fatal to the whole theory"[2]. Of course, now we know more about how such cooperative behaviors could have evolved in our early microbial ancestors, and I have devoted an entire section to this (see section 8.4). To illustrate these evolutionary dynamics in a more accessible way, we only need to look at our own natural history, in which we find exemplified many of the upsides of cooperation.

[2] Darwin C. On the origin of species by means of natural selection, or, The preservation of favoured races in the struggle for life. London: John Murray; 1859.

> "No man is an island, entire of itself [...] Any man's death diminishes me, because I am involved in mankind […]."
> ~ John Donne (1572-1631)

Human beings do not thrive when isolated. Like for many other animals today, group living was essential for survival in early human communities. Sticking together facilitates the defense against predators; tasks can be divided among specialized group members to increase efficiency; and hunting in groups makes it possible to kill prey animals many times our own weight. The underlying principles of this are so strong that similar interactions can even be found among the cells that make up any individual human. We are all composed of individual cells that cooperate to the very extreme and make up one large, multicellular organism. This aggregate of cells we call "body" is too big to eat for most predators on earth. Also, just like members of a community of people, our cells divide labor by specializing on certain - metabolic - tasks, and can take up resources that would be inaccessible for single, isolated cells by killing and digesting whole plants and animals. This is possible because all of our cells share the same genetic makeup: their interests are, evolutionary speaking, completely aligned.

To see how a multicellular organism such as us, one of the many multicellular examples of extreme cooperation, has evolved in the first place, we must go back to the protagonists of the beginning of this tale: microbes. When we look at how bacteria live in nature, they often occur in little groups of related cells. This is mainly because bacteria propagate by dividing into daughter cells that are, genetically speaking, exceptionally close. Additionally, they often co-exist with cells of other strains and species. This leads to a number of stunningly complex interactions that closely resemble those that we see in our own body: bacterial communities can divide labor, they cooperatively open up resources for each other that would be inaccessible for single bacteria,

they can protect themselves from predators and other competing cells, and they can take down organisms many times larger than themselves by cooperating, for example when pathogenic bacteria cause disease in a host organism.

This PhD thesis represents my small contribution to further understanding these tiny, fascinating creatures and the world they so intimately share with us. We owe it to them. After all, we all came from microbes. We would not be alive without microbes, and neither would most other plants and animals. Their small size has led them to be wrongfully neglected over most of the history of biology, but there is still time to compensate for this; if only we continue to push the boundaries, ask the right questions and explore the microbial world with increasingly sophisticated technology.

If we want to learn about ourselves, how we came to be, and how we interact with others, we should have a close look at our microbial ancestors, who colonized the earth long before us, and their descendants living among us, who are our cousins a-million-times removed. Because no microbe is an island, and neither are we.

Further reading

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11 CURRICULUM VITAE

Name: GRANATO
First name(s): Elisa Teresa
Date of birth: 23.04.1988
Nationality: German

EDUCATION

Ph.D. student, Department of Plant and Microbial Biology 6/2013 - present
University of Zurich, Switzerland
Advisor: Prof. Rolf Kümmerli
Title: "Evolution of cooperation and virulence in *Pseudomonas aeruginosa*"

Master of Science (M.Sc.) Microbiology and Immunology 9/2011 – 4/2013
Swiss Federal Institute of Technology, Zurich, Switzerland
Advisor: Prof. Martin Ackermann
Master thesis: "Social aspects of antibiotic resistance in *Escherichia coli* and directed evolution of the β-aminopeptidase BapA"

Bachelor of Science (B.Sc.) Biology 9/2008 – 6/2011
Ludwig-Maximilians-University, Munich, Germany
Bachelor Thesis in Microbiology

EMPLOYMENT

Ph.D. student, Department of Plant and Microbial Biology 6/2013 - present
University of Zurich, Switzerland
Advisor: Prof. Rolf Kümmerli

PUBLICATIONS

Granato ET, Kümmerli R. The path to re-evolve cooperation is constrained in *Pseudomonas aeruginosa*. **bioRxiv** (**2017**) doi: 10.1101/163048

Granato ET, Harrison F, Kümmerli R, Ross-Gillespie A. Do Bacterial "Virulence Factors" always Increase Virulence? A Meta-analysis of Pyoverdine Production in *Pseudomonas aeruginosa* as a Test Case. **Frontiers in Microbiology** (**2016**) 7:1952

Kümmerli R, Santorelli L, **Granato ET**, Dumas Z, Griffin AS, West SA. Co-evolutionary dynamics between public good producers and cheats in the bacterium *Pseudomonas aeruginosa*. **Journal of Evolutionary Biology** (**2015**) 28: 2264–2274

CONGRESS PRESENTATIONS AND RESEARCH SEMINARS

16th Congress of the Europ. Soc. for Evol. Biology, Groningen, Netherlands 8/2017
Oral presentation (accepted abstract)

Department of Zoology, University of Oxford, United Kingdom 1/2017
Oral presentation

116th General Meeting of the American Soc. of Microbiology, Boston, USA 6/2016
Poster presentation

Jeff Gore Laboratory, Massachusetts Inst. of Technology, Cambridge, USA 6/2016
Oral presentation

"BEEES" Seminar, University of Zurich, Switzerland 3/2015
Oral presentation

15th Congress of the Europ. Soc. for Evol. Biology, Lausanne, Switzerland 8/2015
Poster presentation

Evolutionary Medicine Conference, Zurich, Switzerland 7/2015
Oral presentation

15th Intern. Symposium on Microbial Ecology (ISME), Seoul, South Korea 8/2014
Poster presentation

International Workshop in Evolutionary Biology, Guarda, Switzerland 6/2014
Oral presentation

PRIZES, AWARDS, FELLOWSHIPS

Research Fund of the University of Zurich (Forschungskredit) 1/2016 – 2/2017
[86'000 CHF]

ASM Student and Postdoctoral Travel Award 3/2016
[750 USD]

Student Oral Presentation Award (Evol. Medicine Conference, Zurich) 7/2015

Scholarship of the German National Merit Foundation 9/2011 – 4/2013
[12'000 EUR]

Award for graduating in top 10 % of B.Sc. class, LMU Munich, Germany 6/2011